PROCEDURES FOR THE ISOLATION AND IDENTIFICATION OF MYCOBACTERIA

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PROCEDURES FOR THE ISOLATION AND IDENTIFICATION OF MYCOBACTERIA

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Introduction

This manual has been prepared for the laboratorian who is charged with the technical responsibility of isolating and identifying the mycobacteria commonly encountered in clinical specimens. It contains detailed instructions for each procedure.

When more than one procedure is given, that procedure designated as used by the Center for Disease Control (CDC), Mycobacteriology Branch, is the one that has proved to work best for this Branch in its reference laboratory situation. However, other research and reference diagnostic laboratories have found that the alternative procedure is satisfactory for their operation. These specialty laboratories also select from the described identification tests those procedures that best meet their needs.

As a reference for all methods described here, see Chapter 16, "Mycobacterium" by E.H. Runyon, A.G. Karlson, G.P. Kubica, and L.G. Wayne, pages 148-174, in *The Manual of Clinical Microbiology*, 1974, 2nd edition, published by the American Society of Microbiology, Washington, D.C. This chapter also contains an overall philosophy of the laboratory aspects of mycobacteriology as well as a more complete bibliography.

In this 1975 revision of *Procedures for the Isolation and Identification of Mycobacteria*, some of the text of the previous editions has been rearranged to provide a better organization of material. Some few additional species of mycobacteria are described along with their identification characteristics.

In a work of this nature, it is impossible to acknowledge all of those who have, in one way or another, contributed the material, advice, and stimulation needed to accomplish the task. However, I would like to thank the members of the Mycobacteriology Branch of CDC for their criticisms and contributions; and Mr. John P. Gust, Jr., Medical Audiovisual Branch, CDC, for his excellent photographs, suggestions, and cooperation in depicting the colonies and test results shown in this book. I would also like to express my sincere thanks to Dr. Roslyn Q. Robinson, Director, Bureau of Laboratories, and Dr. Phyllis Q. Edwards, Chief, Tuberculosis Control Division, CDC, for their encouragement and support in making this manual possible.

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A. INTRODUCTION

Each laboratory worker must be responsible for his own safety and that of his co-workers.

To avoid accidental infection:

Do not take unnecessary risks.
Use safety equipment properly.
Be prepared to act quickly in case of accident.
Prohibit smoking and eating in the laboratory.

Pulmonary diseases such as tuberculosis are known to be spread by airborne transmission. Many manipulations in the laboratory cause aerosols that may lead to human infection. Some common sources of aerosols are:

Opening specimen containers
Pipetting
Flaming loops
Centrifuges
Shaking machines
Blenders
Syringes
Animal autopsy
Opening lyophilized ampoules

Specially designed suites for tuberculosis bacteriology are of no value unless they are properly used. Proper control depends more on human factors than on laboratory design or the development of new procedures and equipment. Administrators should see that the essential equipment is available and properly used.

Safety measures described here are an accumulation of common sense practices devised by experienced personnel. With careful planning, an efficient routine may be set up without sacrificing safety, accuracy, or speed.

B. PERSONNEL

Personnel should be selected with care. They should be physically and mentally capable. Fatigue, emotional stress, and malnutrition contribute to the development of tuberculosis following exposure.

Before working in the laboratory, all personnel should have (1) a tuberculin skin test, (2) a chest X-ray, and (3) training in safety techniques and procedures.

It is difficult to recommend a uniform program of surveillance testing of tuberculosis laboratory personnel because of the many associated unknowns. The two possible situations are the "safe" laboratory and the "unknown" laboratory.

1. The Safe Laboratory

   A laboratory may be considered safe if all personnel have been meticulously trained in correct laboratory procedures, if all safety equipment (notably the biological safety cabinet) is functioning properly, and if a routine skin testing program has revealed no tuberculin converters among the staff. In such a well-documented situation, tuberculin negative personnel need not be skin tested more often than annually, and X-rays would be required only if an individual exhibited symptoms.

2. The Unknown Laboratory

   Little or nothing is known about the tuberculin conversion rate in the staff of the unknown laboratory, i.e., there is no evidence that safety procedures are adequate or that equipment is functioning properly. In this situation, the following protocol is suggested.

   a. Re-test tuberculin negative personnel at 4-to 6-month intervals, until it is evident that equipment is working properly and staff members are adhering to safety procedures (i.e., no conversions are noted).

   b. Require semi-annual X-rays only for tuberculin positive persons until evidence suggests this is not necessary.

   c. Monitor safety equipment and laboratory air flow to insure their optimal performance.

   d. Discuss safety precautions with laboratory personnel periodically.

If an employee converts from skin test negative to positive, the following should be done:

The employee should be referred to a physician for care or possible treatment.

The employee should check his own personal laboratory habits.

The laboratory should re-evaluate its safety equipment and procedures.

Remember, however, that even the best safety equipment is no substitute for meticulous technique.

C. GUIDELINES

Mycobacterial examinations should be permitted only in a laboratory having an adequate Biological Safety Cabinet (BSC).

1. A Layout for a Mycobacteriology Laboratory

a. DEFINITIONS

(1) Contaminated area(s): Any room(s) in which specimen containers are opened, cultures are prepared or transferreed, and animals are inoculated, housed after inoculation, or autopsied.

(2) Contaminated equipment: ALL equipment used within a contaminated area, e.g., specimen containers, needles, syringes, pipettes, metal trays, discard pans, culture tubes, centrifuges, autopsy equipment, pencils, pens, and culture request slips.

b. GENERAL REQUIREMENTS:

(1) A completely self-contained suite housing all equipment needed for processing specimens from receipt to final report. (See Figure 1.)

(2) A ventilation system engineered to maintain airflow in one direction only, i.e., from a clean area into a less clean area. Keep a continuous check on airflow by means of short tissue paper strips in areas of rapid flow (air duct openings or grills in doors and walls).

(3) An exhaust system (with bacteriologic filters) discharging air to the outside of the building at the roof or side into an area where the possibility of return to the building is minimal.

(4) A separate room, within the suite, designated as the Isolation Room or Contamination Area and containing a biological safety cabinet for the opening of specimens, digestion of sputa, preparation of slides, and inoculation of cultures. (See Figure 2.)

2. Basic Requirement for an Efficient Biological Safety Cabinet (BSC)

No safety cabinet is 100% effective.
There is no substitute for care in performing all techniques.

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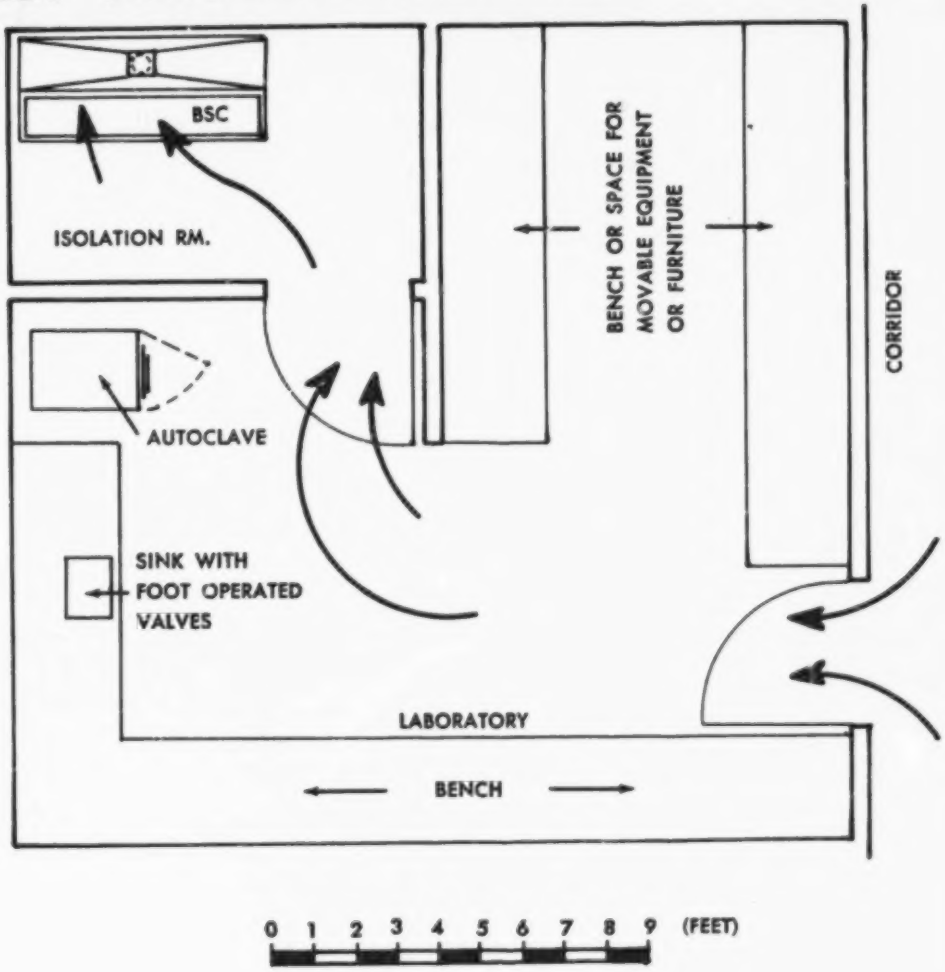

Figure 1. Laboratory Suite .

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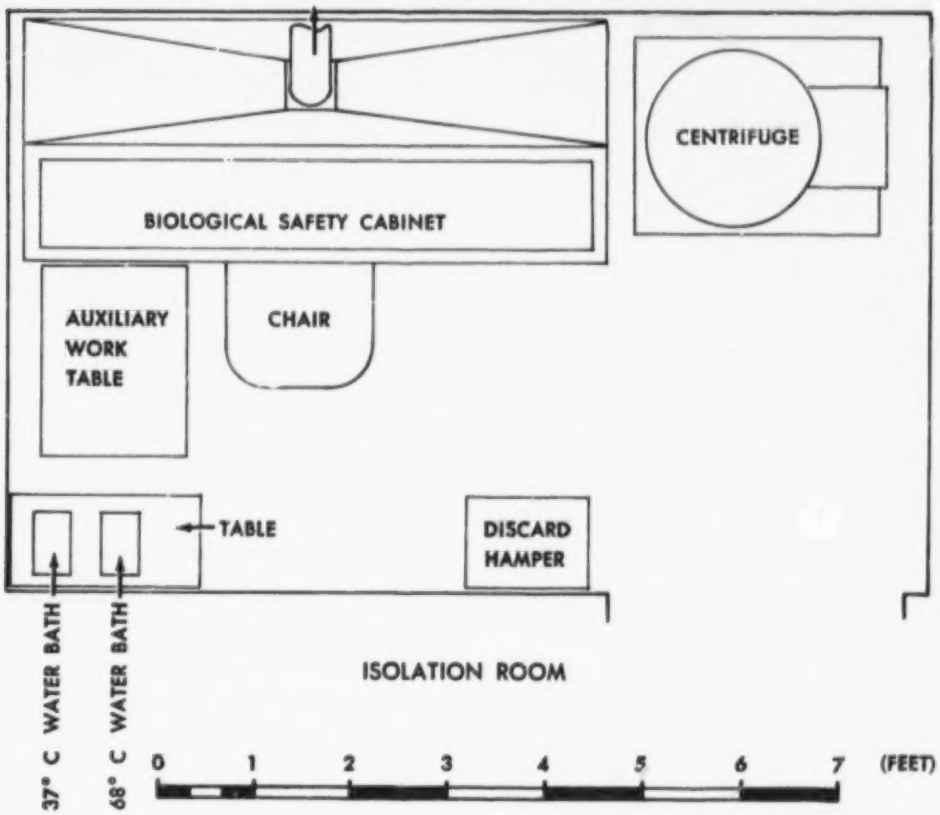

Figure 2. Isolation Room

a. **EXHAUST FAN:**

(1) It should have the capacity to draw a minimum of 50 to 75 lineal feet of air per minute across the entire front opening.

(2) Operate it during all manipulations with specimens, cultures and animals.

(3) Check the airflow at least every 3 months; airflow rate gauges may be mounted on the cabinet to continuously monitor the airflow.

b. **ULTRAVIOLET LIGHTS (USE ONLY BULBS EMITTING RAYS PRIMARILY OF WAVELENGTH 2537 ANGSTROMS):**

(1) These should be mounted within the working area of the cabinet.

(2) Do not operate while the cabinet is in use, since direct or reflected UV rays are injurious to the skin, eyes, and also to the mycobacteria.

(3) Turn on immediately after finishing the work in the cabinet and allow to remain on for a minimum of 2 hours. Continuous operation when the cabinet is not in use costs very little and may be more convenient. **Warning**: Ultraviolet energy has very little penetrating power. These lights serve only as a supplement to proper air flow systems, chemical disinfection and careful techniques.

(4) Clean bulbs at least every 2 weeks with ethyl alcohol to remove residue and dust.

(5) Check at least every 3 months for efficiency of ultraviolet output. UV lamps may be checked with a Westinghouse Model SM-600 Meter or the equivalent. Visible light is NOT a reliable indicator of germicidal ultraviolet output (i.e. 2537 Angstrom wavelength).

3. **General Maintenance**

a. Permit no servicing, cleaning, or checking of isolation room equipment unless a trained technical or professional person is present to insure that adequate safety precautions are observed.

b. When the face-air velocity of the BSC falls below 50 lineal feet (15.24 meters) per minute, it may be possible

to reset the air volume control to increase the air flow rate. If not, new filters must be installed or other adjustments made according to the type of BSC in the laboratory.

c. If filters are to be changed, they must be decontaminated before removal from the filter chamber so that the person who makes the change will be protected. Decontamination may be accomplished by the use of formalin vapors or some other method approved and performed by the engineers.

d. Before major servicing, such as changing filters, decontaminate the entire room by fogging; then wash down the ceiling, walls, floor, and all equipment with a 5% aqueous solution of phenol or an equally effective disinfectant.

e. Let no one enter the Contaminated Area in the event of gross contamination (as by breakage of culture tubes) until the entire area has been decontaminated by individuals within the area, unless an injured person requires emergency care.

4. Other Safety Requirements

a. Foot-operated controls for soap and water at lavatories for handwashing.

b. Aerosol-free safety carriers in centrifuges.

c. Blenders and high-speed mixers with aerosol-free safety screwcap seals. DO NOT use kitchen type blenders for homogenizing infected materials. Beware of aerosols; open only in the BSC.

d. Specimen containers sealed with rubber, Teflon, or other plastic-lined screw caps.

e. Discard pans with covers for all contaminated materials and glassware.

f. An autoclave conveniently located to sterilize contaminated material prior to washing or disposal. Use sterility checks to monitor efficiency.

g. Safety pipetting devices. NEVER mouth pipette.

h. A gauze pad, 4 to 6 thicknesses, or towel. Soak with disinfectant and spread over the work surface in the BSC. This retains invisible spatter during culture manipulations.

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i. A slide warming table for heat fixing smears. Maintain temperature at 65° to 70° C.

j. Racks and baskets for carrying cultures. Tubes and plates accidentally slip from bare hands.

k. A splash proof container into which the contaminated fluids are discarded. This may be specially constructed or assembled. (See Figure 3.)

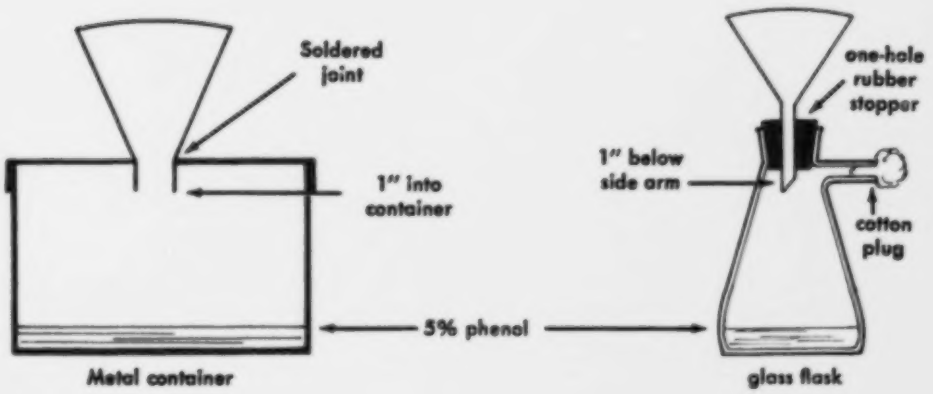

Figure 3. Splash-Proof Containers

l. An alcohol-sand flask to clean large clumps of bacilli from wires and loops before flaming. This prevents spattering of live bacilli which could occur during flaming because mycobacteria are waxy and pop upon heating. (See Figure 4.)

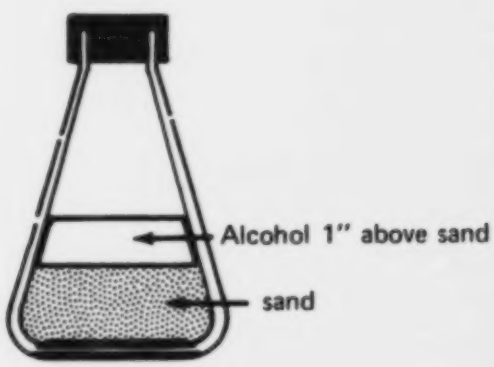

Figure 4. Alcohol-Sand Flask

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m. A culture transfer spade made of heavyweight nichrome wire (18 gauge). Flatten one end to make an area 5 to 10 mm long by 0.5 mm thick. Use this with the alcohol-sand flask. Lightweight wire has a tendency to bend in the sand. (See Figure 5.)

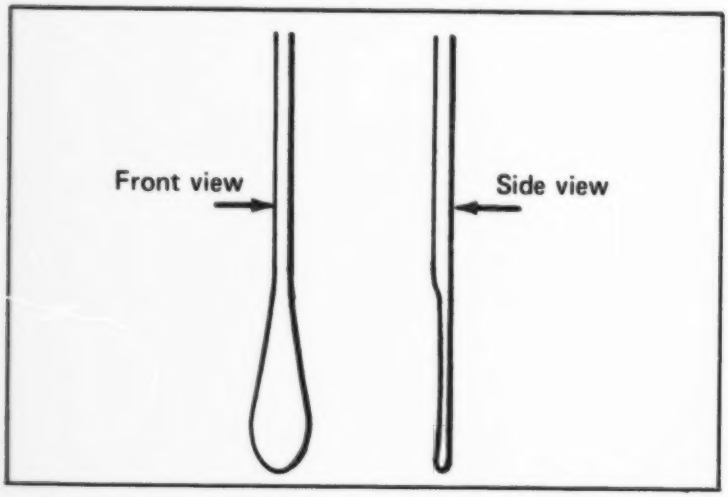

Figure 5. A Transfer Spade

n. BE PREPARED. Have a container of 5% phenol in every room where specimens and cultures are handled or stored. In case of accident, cover broken cultures or specimens with paper or cloth towels, flood with 5% phenol, and let stand for at least one hour, keeping them wet with phenol during this time.

o. Disinfectant for hands, such as 70% alcohol or 2% amphyl. If contaminated materials are handled inadvertently, rinse hands with the disinfectant and wash them well with soap and water.

5. Disinfectants

The killing action of any germicide is never instantaneous; exposure time varies from 30 seconds to 30 minutes or longer. Not all so-called germicides kill tubercle bacilli, for example, quaternary ammonium compounds.

Phenolics and alcohols are effective when properly used. For the tough jobs in discard pans and clean-up, use 5% phenol. Handle only with rubber gloves.

Phenolic soap mixtures are diluted and used according to the manufacturers' instructions.

Alcohols, 70% ethyl or isopropyl, are generally ineffective for clean-up purposes, primarily because they would have evaporated long before their germicidal action was manifested. They may be used as disinfectants for hands.

6. **Protective Clothing**

   a. Wear a protective garment when handling infectious material. The hospital surgical gown is good because the sleeve fits closely at the wrist and does not drag in the work area. The knitted cuff gives a tight seal with gloves and protects the arms from infectious agents. In addition, the hospital gown is easily identified as belonging in the isolation room of the laboratory and not in the general work area.

   b. Wear an effective mask, especially if centrifuges and mixers are not aerosol-free.

   c. Rubber gloves guard against acquiring infection through cuts and abrasions in the skin.

   d. Autoclavable or disposable shoe covers and caps are also available. Do not wear street shoes in the infected area without protecting them with covers.

7. **Preparations Before Entering the Isolation Room**

   a. Assemble all materials and equipment. Label specimens, tubes for processing, media for inoculation, etc., and place them on a cart or table just outside the isolation room door.

   b. Have extra supplies such as media, saline, sterile pipettes, and reagents within easy reach from the isolation room.

   c. Plan other work to do while in the laboratory or while waiting for the centrifuge, e.g., culture-transfers and smears.

   d. Be organized. Keep a check list, if necessary.

   e. Put on protective clothing listed in "6" above.

   ONCE IN THE ISOLATION ROOM, DO NOT LEAVE UNTIL YOUR TASK IS COMPLETED. If you need additional equipment, ask someone to get it for you.

8. **Safety Precautions Within the Isolation Room**

   a. Consider everything within the limits of the Isolation Room as potentially infectious. Let NO ONE enter

without full protective clothing. Organisms on contaminated surfaces may enter the body through cuts and abrasions in the skin.

b. Immediately after each use, place all protective clothing in a covered discard hamper. Autoclave hamper before sending clothing to laundry and before discarding disposable items.

c. Perform ALL work over a gauze pad or towel soaked with 5% phenol solution. When work is completed, place the gauze pad in a covered pan containing 5% phenol solution.

d. Before opening a specimen container, disinfect the outside by wiping it with gauze soaked in 5% phenol solution. (The container may have been contaminated when the specimen was collected, or during transit.)

e. Autoclave inner metal mailing tube before reuse because it may be contaminated.

f. Place laboratory request slips accompanying specimens in a paper bag and autoclave before the slips are handled by the record clerk.

g. Open all tubes and containers with infectious materials within safety cabinet.

h. Any operations involving agitation of specimens or culture should be performed in aerosol-free containers or within the safety cabinet.

i. Use ONLY aerosol-free safety carriers in centrifuge.

j. Heat fix all smears on a slide warmer for at least 2 hours at 65° C; overnight is acceptable. (Shield slides from UV light rays.)

k. BEFORE flaming, clean inoculating loops and spades in an alcohol-sand flask. (See Figures 4 and 5.)

l. Always use safety pipetting devices.

DO NOT mouth pipette under any circumstances.

m. Place ALL materials to be discarded in covered pans containing 5% phenol solution. Autoclave before sending to the washroom.

n. Discard ALL contaminated fluids into a splash-proof container with a small amount of 5% phenol solution.

Autoclave container before disposing of contents.

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o. Place autopsied animals in leak-proof plastic bags, close bags SECURELY, and deposit them in a covered metal container for transport to the INCINERATOR.

p. When work is completed and BEFORE leaving the room:

(1) Place covers on all discard pans. Contaminated material must be in covered containers when brought out of the isolation room.

(2) Wipe the outsides of the discard containers and culture tubes with disinfectant-soaked gauze before placing them outside the isolation room.

(3) Swab around the work area with 2% cresol or 5% phenol. Scrub the area periodically with soap and water to remove disinfectant residue.

(4) Place the gauze pad from the work area in a covered container containing phenol.

(5) Cover the slides on the warmer to protect them from UV light.

(6) Turn on the UV lights in the hood.

(7) Remove protective clothing (MASK LAST) and place in a covered container for autoclaving before laundering or discarding.

UPON LEAVING THE ROOM, scrub hands and arms well with soap and water. Do not wear watches or bracelets while working in the BSC because they interfere with thorough cleansing of hands and arms.

9. **Arrangement of Equipment in a Biological Safety Cabinet**

(See Figure 6.) Convenient for right-handed individuals. Modifications are necessary for other types of Safety Cabinets.

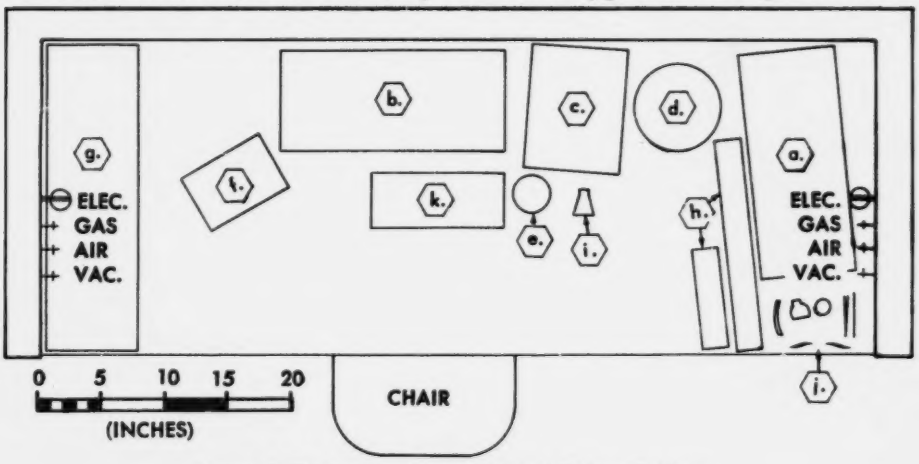

Figure 6. Equipment in a Biological Safety Cabinet

A key to letters on Figure 6 follows:

a. Discard pan for reusable pipettes.
b. Discard pan for test tubes, flasks, etc.
c. Discard container for disposables, plastic, paper, etc.
d. Splash-proof waste container for contaminated liquids (Figure 3).
e. Bunsen burner. The rubber connecting hose should be long enough for the flame to be within easy reach. Avoid over-heating the room by keeping flame low.
f. High-speed mixer to left of worker. Move to rear after processing specimens, if space is needed for work area.
g. Slide warmer to extreme left.
h. Pipette cannisters at right.
i. Alcohol-sand flask for cleaning spade or loop (Figures 4 and 5).
j. Safety pipetting devices, interval timers, pencils, loops, forceps, and other small equipment.
k. Racks for tubes during processing, etc.

NOTE: *This arrangement leaves a clear area immediately in front of the worker for handling specimens. Thus, any drops or invisible spatter are more likely to fall on the gauze, not the work.*

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SECTION II. ISOLATION AND IDENTIFICATION OF MYCOBACTERIA

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A. INTRODUCTION

Because special reagents and expertise are necessary for optimal performance of tuberculosis bacteriology, each laboratory must determine the level of services it can offer and select those laboratory activities needed for it to function at its own level of responsibility.

1. Levels of Service

In 1974 the American Thoracic Society issued a policy statement to the effect that with the decreasing tuberculosis patient load and the increasing complexity of laboratory testing, it is advisable to concentrate the full spectrum of bacteriologic support in only a few laboratories (public or private) in a given community where professional expertise and complete and safe facilities are available.

A brief summary of the recommended levels of service is as follows:

Level	Type Facility	Type Service
I	Physician's Office Outpatient Clinic Small Hospitals	Collect good specimens. Ship to Level II or III Microscopic examination
II	Treatment Center or Selected Laboratory (Hospital or other)	Collect specimens Microscopic examination Isolate organism Identification of *M. tuberculosis* Susceptibiltiy tests to first-line drugs Send isolates to Level III for identification of other mycobacteria
III	Reference Laboratory	All Procedures Level II Complete species identification Susceptibility tests to all drugs

Reference:

Hawkins, J. E., Karlson, A. G., Wayne, L. G. and Wolinsky, E. 1974. American Thoracic Society policy statement on quality of laboratory services for mycobacterial disease. Amer. Rev. Resp. Dis, *110*: 376-377.

2. Daily Work Schedule

Modify according to level of service.

Individual laboratories should select those activities needed for performing the services for Level I, II, or III.

	IN REGULAR LABORATORY	IN BIOLOGICAL SAFETY CABINET
Each Day	Number clinical specimens Label tubes, media, and slides. Prepare reagents. Stain and read slides. Send out reports. Incubate cultures.	Process clinical specimens Make smears. Inoculate media. Do drug tests.
Monday	Read and record: Tween test, 5 and 12 days, tellurite, arylsulfatase.	(Process clinicals.) Add Na carbonate to arylsulfatase.
Tuesday	Read all cultures.* Record: growth rate, number of colonies, morphology, pigment. Set aside cultures for tests. Expose non-chromogenic to light for 1-hour and reincubate. Read MacConkey, 11 days old. Set-up tests to be done on Wednesday.	(Process clinicals.)
Wednesday	Read MacConkey, 5 days old Read NaCl tolerance. Record test results: niacin, nitrate, catalase. Read 1-hour light.	(Process clinicals.) From cultures Tuesday: Inoculate Tween test. Perform niacin, nitrate, catalase tests. Make other necessary culture transfers.
Thursday	Read cultures, if necessary. Read Tween test, 24 hours. Set-up tests to be done on Friday Check reagent supply.	(Process clinicals.)
Friday	Read Tween test, 9 days. Check cultures inoculated during the week for rapid growers.	(Process clinicals.) Transfer to liquid medium: Rapid Growers Slow Growers for tellurite Inoculate 7-day old liquid cultures to arylsulfatase, MacConkey, NaCl medium. Add tellurite to 7-day culture.

* When cultures must be read more than 1 day a week, perform in vitro tests on Monday and/or Wednesday.

SECTION II B. SPECIMENS AND TRANSPORTATION

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B. SPECIMENS AND TRANSPORTATION

Specimens must be collected in clean, sterile containers and sent directly to the laboratory for immediate processing. If possible, specimens should be refrigerated during transit to the laboratory. Refrigeration discourages the multiplication of organisms that reproduce at room temperature and sometimes make decontamination of the specimen impossible in the laboratory.

The specimen container must be clearly labeled with the patient's name and/or hospital number and the date the specimen was taken.

1. Types

a. SPUTUM

These specimens should be a series of 3 to 5 single early morning samples. A volume of 5 to 10 ml is adequate for each sample. There is no advantage in collecting a larger volume. Any volume, no matter how small, should be processed. Keep a record of volumes less than 2 ml and enter this information on the report form. It may be of value to the physician.

b. INDUCED (or NEBULIZED) SPUTUM

These specimens are usually very watery and should be labeled as "induced" so that they will not be mistaken for saliva.

c. GASTRIC LAVAGE

These specimens must be processed within 4 hours after collection.

d. URINE

A series of single, mid-stream specimens, voided in the early morning, should be submitted, rather than a 24-hour pooled specimen.

e. TISSUE

Any tissue to be processed must be collected aseptically and transported to the laboratory at once. When a delay is necessary, freeze the tissue and transport to the laboratory in the frozen state.

f. **OTHER TYPES**

These include aseptically collected specimens, such as blood, pleural fluid, bronchial washings, pus, joint fluid, and laryngeal swab.

2. **Transportation**

a. The shipment of diagnostic specimens such as urine, sputum, and tissue and of cultures of etiologic agents, which includes all *Mycobacterium* spp., is subject to the packaging and labeling requirements of the Interstate Quarantine regulations (Federal Register, Title 42, Chapter 1, Part 72, revised July 30, 1972). Compliance with the requirements of the regulation is the responsibility of the shipper. Failure to comply with the applicable requirements subjects violators to penalty provisions which include fines and/or imprisonment.

Diagnostic specimens must be packaged to withstand leakage of contents, shocks, pressure changes, and other conditions incident to ordinary handling in transportation. The "ETIOLOGIC AGENT/BIOMEDICAL MATERIAL" label (described below) *must not* be affixed to shipments of *diagnostic* specimens.

b. Etiologic agents are subject to additional specific containment packaging and labeling requirements. Cultures of *Mycobacterium* should be shipped to reference laboratories on solid medium in screw-cap tubes. Petri dish cultures and cultures in liquid medium must not be shipped.

Requirements for packaging etiologic agents are found in Section 72.25, paragraph (c) subparagraph (1) of the Interstate Quarantine regulations, as follows: *Volume less than 50 ml.* Material shall be placed in a securely closed, watertight container (primary container [test tube, vial, etc.]) which shall be enclosed in a second, durable watertight container (secondary container). Several primary containers may be enclosed in a single secondary container, if the total volume of all the primary containers so enclosed does not exceed 50 ml. The space at the top, bottom, and sides between the primary and secondary containers shall contain sufficient nonparticulate material to absorb the entire contents of the primary container(s) in case of breakage or leakage. Each set of primary and secondary containers shall then be enclosed in an outer shipping container constructed of corrugated fiberboard, cardboard, wood, or other material of equivalent strength. See Figure 7A.

Subparagraph (2) describes packaging of *volumes of 50 ml or greater.* This description is similar to (1) except that the volume in a single primary container may not be over 500 ml and the total volume enclosed within the outer shipping container may not exceed 4000 ml.

Subparagraph (3) states that if dry ice is used as a refrigerant, it must be placed between the secondary and outer container in such a way that, as the dry ice sublimates, the secondary container does not become loose inside the outer container.

Department of Transportation regulations prohibit the shipment of more than 50 ml of an etiologic agent on a passenger aircraft. A volume greater than 50 ml may be carried by air freight provided it is properly packaged and the total volume within a single outer container does not exceed 4000 ml.

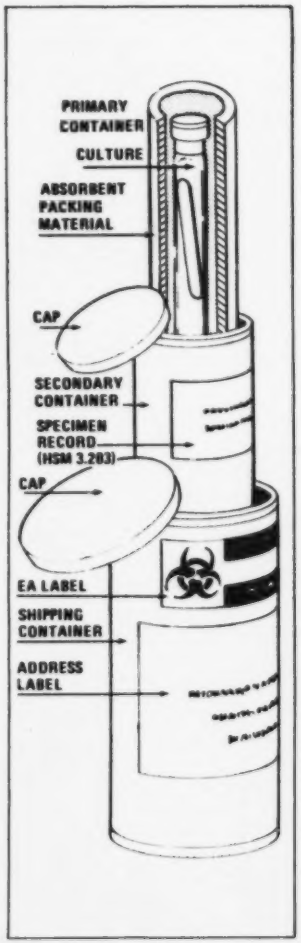
An "ETIOLOGIC AGENT/BIOMEDICAL MATERIAL" label must be shown on the outer container. These labels are available commercially (See Figure 7B); they should measure 2 inches high by 4 inches long, and be printed in red on a white background. Shippers of etiologic agents must be familiar with the above regulations that govern packaging, labeling, and shipment of these products in interstate commerce.

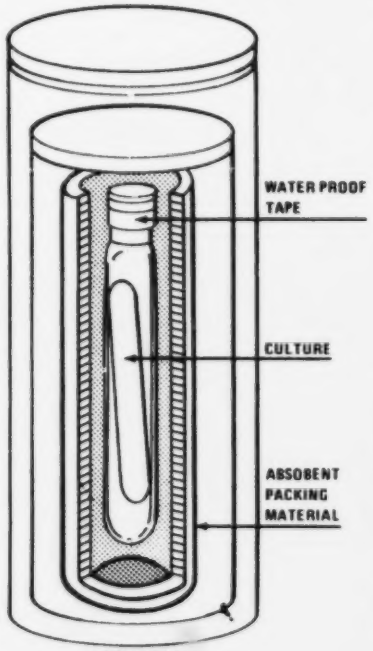
Questions regarding this regulation should be addressed to:

Center for Disease Control
Attention: Biohazards Control Officer
Office of Biosafety
Office of Center Director
Atlanta, Georgia 30333
Telephone 404-633-3311, Ext. 3883.

Figure 7A

PACKAGING AND LABELING OF ETIOLOGIC AGENTS





CROSS SECTION OF PROPER PACKING

The Interstate Quarantine Regulations (42 CFR, Part 72.25 Etiologic Agents) was revised July 31, 1972 to provide for packaging and labeling requirements for etiologic agents and certain other materials shipped in interstate traffic.

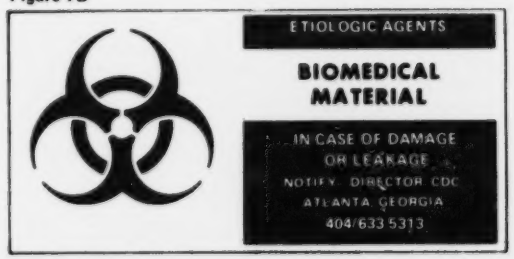
Figure 7A diagrams the packaging and labeling of etiologic agents in volumes of less than 50 ml. in accordance with the provisions of subparagraph (C) (1) of the cited regulation. Figure 7B illustrates the color and size of the label, described in subparagraph (C) (4) of the regulations, which shall be affixed to all shipments of etiologic agents.

For further information on any provision of this regulation contact:

Center for Disease Control
Attn: Office of Biosafety
1600 Clifton Road
Atlanta, Georgia 30333

Telephone: 404 - 633 3311

Figure 7B



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SECTION II C. ISOLATION PROCEDURES AND INCUBATION

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C. ISOLATION PROCEDURES AND INCUBATION

INTRODUCTION

Use an effective but gentle technique. Follow the procedure precisely, because overexposure to any decontaminating agent will kill mycobacteria as well as the undesirable organisms.

The N-acetyl-L-cysteine-sodium hydroxide (NALC) technique of Kubica and Dye (4) is used at CDC. This technique utilizes a mucolytic agent, N-acetyl-L-cysteine, for digestion and sodium hydroxide for decontamination. The sodium citrate included in this mixture exerts a stabilizing effect on the acetylcysteine because of its ability to bind, by chelation, many heavy metal ions that may be present in the specimen. (5).

The technique of Wayne and Krasnow (7) utilizes trisodium phosphate for 1 hour to homogenize the specimen and incorporates the quaternary ammonium compound, benzalkonium chloride (Zephiran®), to eliminate contaminants. A buffer-wash neutralizes the benzalkonium chloride (3).

Contaminants such as Pseudomonas are not killed by the usual alkaline system. To process specimens from those patients whose cultures are consistently contaminated with Pseudomonas, use 5% oxalic acid (2). Also, contaminants in thin, clear specimens, such as urine, are more readily destroyed by an acid such as sulfuric than by an alkali (8).

PREPARATION

Label all media, slides, centrifuge tubes, etc., with appropriate numbers. Culture tube labels should also show the date of inoculation. Assemble all specimens, solutions, and other supplies; place them on a cart or table beside the door to the isolation room. THEN don protective clothing, enter the isolation room, and transfer the materials into the work area. DO NOT leave the work area until the work has been completed. Have an assistant on call to supply any emergency needs.

1. N-Acetyl-L-Cysteine-Sodium Hydroxide (NALC-NaOH)

a. EQUIPMENT AND SUPPLIES

Sterile centrifuge tubes with water-tight screw caps, 50 ml.

(5) Tighten caps of centrifuge tubes. Beginning with the first specimen in the series, mix each specimen on the Vortex until liquefied. This may take from 5-20 seconds per specimen.

(6) Set timer for 15 minutes as soon as 1 or 2 specimens have been liquefied.

(7) Let each specimen stand for 15 minutes.

Work at such speed that each specimen stands for 15 minutes and not more than 20 minutes before the diluent is added.

(8) Fill each tube with pH 6.8 buffer or water. This should be at least 30 ml in a 50 ml centrifuge tube. Tighten cap and swirl by hand to mix. Proceed at once to step (9).

(9) Centrifuge at 3000 rpm (1800-2400 x *g*) for 15 minutes.

(10) Pour off all of the supernatant into a splash-proof can containing a disinfectant. Flame lips of tubes to prevent contamination, or wipe lip of tube with a cotton pledget soaked with 5% phenol. Use one pledget per specimen. Do not let any phenol run down the inside of the specimen tube.

(11) With a sterile pipette, add 1 or 2 ml of 0.2% bovine albumin or sterile distilled water to each sediment. Shake gently by hand to mix because vigorous shaking creates aerosol.

NOTE: *Adjusting the pH of the sediment is not necessary because of the well-buffered media. But do not over-inoculate because this interferes with the buffer action and results in poor growth.*

(12) Using a sterile capillary pipette, mix sediment and make a 1:10 dilution by adding 0.5 ml (10 drops) to 4.5 ml sterile water or saline. Mix with the pipette.

(a) Place 0.1 ml (or 2 drops) of the 1:10 dilution onto the surface of one half of a bi-plate of 7H-10 medium, and place 2 drops on the surface of each of 2 tubes of egg-base medium.

(b) Place 2 drops of the undiluted sediment on the second half of the bi-plate of 7H-10 and 2 drops on each of 2 tubes of egg-base medium.

(c) With a bent glass rod, spread inoculum over the surface of the 7H-10*. (Spread the 1:10 dilution and then the undiluted.) Inocula on plates may be left without spreading. In such cases 3 drops per half-plate are recommended.

(d) Make a smear of the undiluted sediment by spreading a drop over an area of 1 x 2 cm on the microscope slide. Discard pipette. Place slides on an electric slide warmer at 65° - 75° C for 2 hours (overnight is acceptable).

(13) Refrigerate excess undiluted sediment for direct drug test. (See page 100.)

Laryngeal Swab

(1) With sterile forceps, transfer the swab to a sterile centrifuge tube.

(2) Add 2 ml sterile distilled water or saline.

(3) Add 2 ml NALC-NaOH.

(4) Swirl on Vortex mixer.

(5) Let stand 15 minutes to decontaminate.

(6) With sterile forceps, remove swab from centrifuge tube.

(7) Fill the tube with sterile buffer or water and proceed as for sputum.

Gastric Lavage

Mucoid gastric lavage:

(1) To 20-50 ml of gastric lavage add 50-100 mg ("pinch") of NALC powder.

(2) Mix by hand or with test tube mixer as for sputum.

(3) Centrifuge at 3000 rpm (1800-2400 x *g*) for 30 minutes.

(4) Decant supernatant fluid and resuspend sediment in 2-5 ml of sterile distilled water.

(5) Add an equal volume of NALC-NaOH and proceed as for sputum.

Fluid gastric lavage:

(1) Centrifuge entire specimen for 30 minutes.

*A disposable bent glass rod is quickly made by heating the capillary portion of a pipette in the gas flame to form a right angle.

(2) Decant and resuspend in 2-5 ml of sterile distilled water. Pool sediments if more than one container is used for centrifugation.

(3) Add an equal volume of NALC-NaOH and proceed as for sputum.

Tissue

(1) Grind with sterile tissue grinder in sterile 0.85% saline or 0.2% bovine albumin. When tissue contains mucus (e.g., lung), add a pinch of N-acetyl-L-cysteine.

(2) If collected aseptically, inoculate directly to both liquid and solid media.

(3) Smear liquid culture once a week until acid-fast bacilli are seen. Inoculate solid media with this liquid culture if necessary.

(4) When tissue is not collected aseptically, place in a tube and process as sputum.

Blood

(1) Add 10 ml aseptically collected blood to 50 ml of a Tween liquid medium such as Dubos or Middlebrook 7H-9.

(2) Incubate at 35°-37° C.

(3) Smear once a week until acid-fast bacilli are found.

(4) Then inoculate solid media with this liquid culture.

Other Body Fluids

Muco-purulent materials:

(1) Handle as for sputum when volume is 10 ml or less.

(2) Handle as for mucoid gastric lavage when volume is more than 10 ml.

Fluid Materials:

(1) If collected aseptically, centrifuge and inoculate sediment directly onto culture media.

(2) If not aseptically collected:

(a) Handle as for sputum when volume is 10 ml or less.

(b) Handle as for fluid gastric lavage when volume is more than 10 ml.

(c) Make a smear of the undiluted sediment by spreading a drop over an area of 1 x 2 cm on the microscope slide. Discard pipette. Place slide on an electric slide warmer at 65°-75° C for 2 hours (overnight is acceptable).

2. **Oxalic Acid (For sputum contaminated with Pseudomonas)**

   a. **SOLUTIONS (FOR PREPARATION, SEE APPENDIX D.)**

   5% oxalic acid.
   4% sodium hydroxide.
   Sterile physiological saline.

   b. **PROCEDURE**

   (1) Add equal volume 5% oxalic acid to the specimen.

   (2) Mix on test tube mixer. Let stand 30 minutes. Shake occasionally.

   (3) Add sterile saline to lower the specific gravity and dilute the acid.

   (4) Centrifuge at 3000 rpm (1800-2400 x g) for 20 minutes.

   (5) Decant. Retain sediment.

   (6) Add an indicator and neutralize with 4% NaOH.

   (7) Inoculate media as described for sputum, step (12).

   (8) Make smear and stain for acid-fast bacilli.

3. **Sulfuric Acid (For Urine Specimens)**

   a. **SOLUTIONS (FOR PREPARATION, SEE APPENDIX D.)**

   Sterile distilled water.
   4% sulfuric acid.
   4% sodium hydroxide.

   b. **PROCEDURE**

   (1) Centrifuge entire specimen for 30 minutes at 3000 rpm. (May need several tubes.)

   (2) Decant supernatant fluid and pool sediments. If volume is small, it is permissible to add 1 or 2 ml of sterile water.

   (3) Add an equal volume of 4% H_2SO_4 to the sediment.

   (4) Mix and let stand for 15 minutes.

(5) Fill the tube with sterile water.

(6) Centrifuge. Decant supernatant fluid.

(7) Add an indicator and neutralize with 4% NaOH.

(8) Inoculate media as described for sputum, step (12).

4. **Zephiran-Trisodium Phosphate (Z-TSP)**

a. **SOLUTIONS (FOR PREPARATION, SEE APPENDIX D.)**

Zephiran-trisodium phosphate.
Neutralizing buffer, pH 6.6 (sterilize by autoclaving).

b. **PROCEDURE**

(1) Add to the specimen a volume of Z-TSP equal to the volume of the specimen.

(2) Agitate vigorously on a mechanical shaker for 30 minutes.

(3) Let stand for 20-30 minutes without additional shaking.

(4) Transfer to a screw-cap centrifuge tube, 50 ml.

(5) Centrifuge at 3000 rpm (1800-2400 x *g*) for 20 minutes.

(6) Decant the supernatant fluid into a splash-proof can.

(7) Add 20 ml of the neutralizing buffer. Mix well.

(8) Centrifuge again for 20 minutes.

(9) Discard the supernatant fluid.

(10) With a sterile capillary pipette, mix the sediment and inoculate 3 drops to each tube or plate of media as described for sputum, step (12).

5. **Animal Inoculation (8)**

Guinea pig inoculation* may be profitable as an adjunct to culture with certain specimens, e.g.:

a. Sputum from patients whose specimens are consistently contaminated on culture.

b. Urine from patients with repeated negative cultures in whom tuberculosis is still suspected.

c. Other body fluids where organisms may be few in number.

*INOCULUM: 1-2 ml of neutralized sediment, administered subcutaneously in the right groin of the guinea pig.

Generally, specimens for animal inoculation should be discouraged or referred to specialized laboratories. Multiple specimens and modern *in vitro* techniques are usually sufficient for isolation and identification. All mycobacteria that produce disease in man do not produce progressive disease in the laboratory animal, e.g.:

a. *M. tuberculosis* resistant to isoniazid.
b. Clinically significant mycobacteria other than tubercle bacilli.

6. Incubation of Cultures

a. Plates of inoculated media may stay for a few hours at room temperature before they are sealed in CO_2 permeable plastic bags for incubation. Keep shielded from light (6).

b. Temperature: 35°, ±2°C, not to exceed 37°C. However, all cultures from skin lesions must also be incubated at a temperature not to exceed 33°C. It could be *M. marinum* or *M. ulcerans* infection.

c. Atmosphere: 5%-10% carbon dioxide in air (1).

d. Medium in tubes:

Place in a slanted position with the screw cap loose for at least 1 week to permit exposure to carbon dioxide for the initiation of growth. Tighten caps to prevent dehydration, and stand tubes upright if space is a problem.

e. 7H-10 medium in plates:

Place plates medium-side down in a plastic bag; polyethylene allows the passage of carbon dioxide into the bag. Seal the bag with tape or a heat-sealing apparatus, or fold the top over 2 or 3 times and staple. Squeeze the air out of the bag before sealing.

f. If the accumulation of moisture during incubation is a problem, some of the following suggestions may be helpful:

(1) Avoid inoculating a wet medium surface. Take plates out of the refrigerator on the day before they are to be inoculated and leave them at room temperature in a clean, dark area.

(2) After inoculation, shield plates from contaminating air currents and direct and indirect daylight. Leave

the plates for several hours before placing them in the incubator. This permits some of the inoculum fluid to evaporate or be absorbed.

(3) During incubation, follow these recommendations:

(a) Incubate plates in individual bags. This facilitates weekly reading of plates. Furthermore, moisture tends to accumulate in the plate on the bottom of the pile, and rotation helps prevent this.

(b) If several plates are incubated in a large bag, stack them no more than 6 high. Pressure of the upper plates may "seal" moisture in lower plates and also interfere with the circulation of CO_2.

(c) Incubate plastic plates on an "insulating" surface. Plastic plates in direct contact with metal shelves seem to collect more moisture than those "insulated" from the metal shelving. Styrofoam (one-half inch) is a satisfactory insulator; also, corrugated cardboard serves the purpose.

(d) Avoid incubating plates on shelves adjacent to the door. Alternate warm and cool air currents, created by opening and closing the door, cause "sweating" and the collection of moisture within the plastic bags.

(e) Make sure that the air within the incubator is circulating properly.

(4) When reading plates stacked in large plastic bags, open the bags immediately after removing them from the incubator. Plates incubated singly in transparent plastic bags may be read without opening the bag.

(5) Condensate on the inside of plate lids interferes with reading cultures. To avoid this, have a warming pad* adjacent to the microscope and place plates on it lid-side down.

*An electric heating pad is satisfactory.

g. For Maximum growth on culture media:

(1) Follow directions precisely during decontamination procedure. Overexposure to the chemicals may "damage" mycobacteria and retard their growth.

(2) Make sure there is no pH imbalance. Such an imbalance may be due to:

(a) Failure to centrifuge sufficiently to pack the sediment so that all the supernate can be decanted, or

(b) A too heavy inoculum, which interferes with the buffering action of the medium.

(3) Make sure the supply of CO_2 in the incubator is sufficient.

(a) The recommended concentration of CO_2 is 5%-10%. Concentrations above 40% may be toxic.

(b) A Fyrite CO_2 gas analyzer, or some other means of determining the concentration of CO_2, should be used daily for testing the atmosphere of the incubator.

(c) Be certain the plastic bags in which the plates are incubated are of polyethylene which permits the circulation of CO_2; Mylar bags are *not* CO_2 permeable.

(d) Many strains of mycobacteria are dependent upon CO_2 and will not grow without it.

(4) In preparation:

(a) Use good quality ingredients and follow directions.

(b) Do not overheat medium during preparation. Monitor temperature of autoclaves and inspissators.

(c) Do not expose the medium to direct daylight during or after preparation. This is especially important for 7H-10 and 7H-11 (6).

(d) Plates of agar medium should contain 20 ml; otherwise the medium may be too thin and dry out during storage or incubation.

(5) Since some strains of mycobacteria grow more slowly than others and need an extra week or two of

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incubation to produce visible colonies, do not discard cultures as negative until after at least 6 to 8 weeks.

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SECTION II D. MICROSCOPY AND STAINING

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D. MICROSCOPY AND STAINING

1. Ziehl-Neelsen (Z-N)

a. REAGENTS

(1) Carbol fuchsin

(Prepare a saturated alcoholic solution by dissolving 3.0 g basic fuchsin in 100 ml of 95% ethyl alcohol.)
Saturated alcoholic basic fuchsin .....10.0 ml
5% aqueous solution of phenol
(liquefied crystals) .....90.0 ml
(Prepare by weighing 5.0 g phenol crystals, melt with gentle heat, add 100 ml H_2O.)

(2) Acid alcohol

Ethyl alcohol (95%) .....97.0 ml
Concentrated hydrochloric acid .....3.0 ml

(3) Counterstain

Methylene blue chloride .....0.3 g
Distilled water .....100.0 ml

b. PROCEDURE

(1) Prepare smear, using a 3mm loop to spread a loopful over an area of 2-3 square cm or smear a drop on the slide with a pipette.

(2) Fix smears on an electric slide warmer at 65° to 75°C for 2 hours or use a Bunsen-flame and pass the slide 3 times slowly over its cone of heat. Do not scorch.

(3) Place a piece of filter paper over the smear. This holds the carbol fuchsin on the slide.

(4) Cover the filter paper with carbol fuchsin and heat the slide to steaming with a Bunsen flame or an electric staining rack as follows:

(a) If a Bunsen flame is used, heat gently to steaming (DO NOT BOIL) and allow to stand 5 minutes without further heating. If the slide dries, keep it moist by adding more carbol fuchsin without additional heating.

(b) If an electric staining rack is used, place slides on the rack, turn on the switch, and allow to

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stain for 15 minutes. (If rack has been pre-heated, 7 to 10 minutes is sufficient.)

(5) Remove paper strips and wash slides with tap water. Drain.

(6) Decolorize with acid alcohol until no more color appears in the washings (about 2 minutes). Thick smears may require longer, but do not over-decolorize, since some mycobacteria may lose their acid-fastness if this is done.

(7) Wash with distilled or tap water. Drain.

(8) Flood slide with methylene blue counterstain for 1-2 minutes.

(9) Wash with distilled or tap water. Drain.

(10) Dry in the air or over gentle heat. Do not blot.

(11) Examine smear with oil immersion lens, taking care to wipe the lens well after each examination, especially if the smear is positive. Three long lines the length of the smear or nine short lines the width of the smear are examined.
The bacilli are stained red and the background material is stained blue. (See Figure 8.)

Figure 8. Ziehl-Neelsen Stain of Acid-Fast Bacilli in Digested Sputum (about 1000 X magnification)

2. Kinyoun

a. REAGENTS

(1) Kinyoun carbolfuchsin

Basic fuchsin .....4.0 g
Ethyl alcohol (95%) .....20.0 ml
Dissolve and add slowly while shaking
Distilled water .....100.0 ml
Liquefied phenol (melted crystals) .....8.0 g

(2) Acid-alcohol

Ethyl alcohol (95%) .....97.0 ml
Concentrated hydrochloric acid .....3.0 ml

(3) Counterstain

Methylene blue chloride .....0.3 g
Distilled water .....100.0 ml

b. PROCEDURE

(1) Heat-fix smears on slide warmer at 65° to 75° C for 2 hours or overnight.

(2) Cover smear with Kinyoun carbolfuchsin.

(3) Stain for 5 minutes. No heat is necessary.

(4) Rinse off stain with distilled water.

(5) Decolorize with acid-alcohol for 3 minutes.

(6) Rinse with distilled water.

(7) Repeat decolorization for 1-2 minutes or until no red appears.

(8) Rinse with distilled water.

(9) Flood slide for 3-4 minutes with methylene blue or other appropriate counterstain.

(10) Rinse off stain with water.

(11) Air dry (do not blot) and read as described in b (11) under Ziehl-Neelsen, page 34.

3. Fluorochrome

This stain employs dyes which fluoresce and which stain acid-fast organisms. The nonspecific background fluorescence of the tissue debris may be suppressed by a counterstain such as potassium permanganate or it may become a contrasting background when acridine orange is used as the counterstain.

a. **REAGENTS**

(1) Stain

(a) Dissolve completely 0.1 g Auramine O in 10 ml of 95% ethyl alcohol.

(b) Mix 3 g phenol crystals with 87 ml distilled water. Mix (a) and (b).

(c) Store in a tightly stoppered brown bottle away from heat and bright light. NOTE: Turbidity which may develop upon standing does not affect the staining reaction.

(2) Decolorizing solution (0.5% acid-alcohol)
To 100 ml of 70% ethyl alcohol, add 0.5 ml of concentrated hydrochloric acid.

(3) Counterstain
Use either (a) or (b)

(a) Potassium permanganate
Dissolve 0.5 g potassium permanganate in 100 ml distilled water. Store in a brown bottle.

(b) Acridine orange
Dissolve 0.01 g of anhydrous dibasic sodium phosphate (Na_2HPO_4) in 100 ml distilled water. Then add 0.01 g acridine orange and dissolve. Store in a tightly stoppered brown bottle.

b. **PROCEDURE**

(1) Heat-fix smears on a slide warmer at 65° to 75° C for 2 hours *or* overnight.

(2) Flood smear with Auramine O stain. Let stand at room temperature for 15 minutes. Do not heat.

(3) Rinse with tap water. Drain.

(4) Decolorize with acid-alcohol for 2 minutes.

(5) Rinse with tap water. Drain.

(6) Flood smear with counterstain (a) or (b) for 2 and not more than 4 minutes.

(7) Rinse with tap water. Drain.

(8) Air-dry. DO NOT BLOT.

(9) Read as soon as possible after staining.

c. **MICROSCOPIC EXAMINATION**

(1) Examine with fluorescence microscopy equipment and techniques. A BG-12 or a 5113 primary filter with an OG-1 barrier filter system has proved very satisfactory.

(2) With this system acid-fast organisms emit a bright yellow fluorescence.

With the potassium permanganate counterstain, the nonspecific fluorescing debris is usually a pale yellow, quite distinct from the bright yellow acid-fast organisms.

With the acridine orange counterstain, the background is stained red to orange, which sharply contrasts with the bright yellow acid-fast organisms.

(3) Smears may be scanned rapidly with a 10X objective. Occasionally it may be necessary to use the 40X or 45X objective to ascertain actual bacterial morphology. (Figure 9 illustrates fluorochrome stained bacilli.)

(4) Confirm all positive smears with Ziehl-Neelsen stain. The fluorochrome smear may be overstained with Z-N after examination without removing the auramine stain. If overstained with Z-N, however, the smear is no longer satisfactory for fluorescent examination.

4. Reporting Smears.

Reports should state the staining method used and the number of acid-fast bacilli seen on the smear.
The American Lung Association recommends the following method:

Number of Bacilli	Report
0	No acid-fast bacilli found.
1-2 in entire smear	Report number found and request repeat specimen.
3-9 in entire smear	Rare or +
10 or more in entire smear	Few or ++
1 or more per field	Numerous or +++

A. Observed at 100 X magnification

B. Observed at 450 X magnification

Figure 9. Fluorochrome Staining of Acid-Fast Bacilli in Digested Sputum

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5. Microscopy without Culture

Sputum specimens may be treated with a 5% solution of sodium hypochlorite (commercial Clorox). This makes most of the bacilli nonviable, yet they remain acid-fast. Such a technique is useful when a smear examination is required even though a biological safety cabinet is not available for concentrating and culturing the specimen by conventional procedures.

a. Place equal volumes of sputum and Clorox in a screw-cap test tube.

b. Shake until liquefied and let stand 15 minutes (or place on a mechanical shaker for 15 minutes).

c. Centrifuge at 3000 rpm for 15 minutes.

d. Decant at once, draining off excess liquid.

e. Smear sediment and stain as desired.

NOTE: *Clorox will result in sterilization of sputum within 15 minutes and, subsequently, will cause disintegration of the bacilli if allowed to act too long; therefore smears should be made, stained, and examined promptly.*

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SECTION II E. CULTURE READING AND IDENTIFICATION

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E. CULTURE READING AND IDENTIFICATION

1. Introduction

Mycobacterium tuberculosis is the most common species of mycobacteria isolated from patients suspected of having tuberculosis. Acid-fast bacilli other than *M. tuberculosis* may also occur in patient specimens. Some of these bacilli may be implicated in disease. It is therefore imperative that *M. tuberculosis* be specifically identified as such and that the potential clinical significance of the other mycobacteria be determined.

Most of the mycobacteria in the Runyon Groups have been given species status and, consequently, species names. In most cases, it is possible to use legitimate names rather than the indefinite Runyon Group classification. The use of species names will assure that only one label is applied to a given organism, thus preventing confusion. Table I lists the most commonly encountered species.

Table I
Potential Clinical Significance of Various Mycobacteria

Strict or Potential Pathogen	Rarely or Probably Not	Formerly Runyon Group
M. tuberculosis *M. bovis* *M. ulcerans** *M. africanum**		
M. kansasii *M. marinum* *M. simiae**		I
M. scrofulaceum *M. szulgai** *M. xenopi*	*M. gordonae* *M. flavescens*	II
M. avium *M. intracellulare*	*M. terrae* complex *M. triviale* *M. gastri*	III
M. fortuitum *M. chelonei*	*M. vaccae* *M. smegmatis* *M. phlei*	IV

*See Section E.5. "Unusual or Rarely Encountered Species"

2. Culture Reading

Careful attention to detail yields valuable information for identification of cultures. A uniform method of recording and filing laboratory data is essential for culture identification and serves as a safeguard when questions arise concerning a report. Cultures represent people; therefore accuracy is imperative.

Colonies and growth rates described here were observed on Lowenstein-Jensen (L-J) and Middlebrook 7H-10 media.

a. METHOD

When reading cultures on egg-base medium, use a hand lens or other magnifier at 3X-10X.

Plates of transparent agar-base medium are inverted on the stage of a dissecting microscope. Use 10X-50X magnification and transmitted light, with the source below the stage so that it shines through the medium. By this method, colonies may be detected at an early stage of growth, the morphology of mature colonies noted, and the presence of minute colonies of the more slowly growing strains observed.

b. TIME

Read all cultures 5-7 days after inoculation and once a week thereafter for 6-8 weeks before discarding as negative.

It is wise to check "negatives" under magnification for minute colonies of slow-growing strains. Drug-resistant *M. tuberculosis* and the species *M. xenopi* and *M. bovis* are noted for being slow in forming macroscopically visible colonies. *M. ulcerans,* when suspected, should be incubated at 30° - 33° C for prolonged time periods because of extremely slow growth (6-8 weeks or more).

c. OBSERVATIONS TO RECORD

(1) Growth rate. This may be defined as the number of days required for colonies to become visible without magnification. For this reason it is helpful to include the "date inoculated" on the label of the culture.

Rapid growers have mature colonies within 7 days. Slow growers require more than 7 days for mature colony forms.

(2) Number of colonies. Record as follows:

No colonies	= Negative	
Less than 50 colonies	= Actual Count	
50-100 colonies	= +	(1+)
Approximately 100-200 colonies	= ++	(2+)
Almost confluent (200-500)	= +++	(3+)
Confluent (more than 500)	= ++++	(4+)

(3) Pigment production.

Colonies that are white, cream or buff are described as nonpigmented or nonchromogenic (NC).

Colonies that are lemon yellow, orange or red are described as pigmented or chromogenic (Ch). Intermediate colorations such as pink, pale yellow or tan sometimes occur, and these are recorded as observed. Such cultures generally are regarded as nonphotochromogens unless the pigment becomes more intense upon exposure to light.

(a) Photochromogens are those cultures whose colonies are non-pigmented when incubated in the dark, but after they are exposed to light for 1 hour and reincubated they become pigmented (Figure 10).

(b) Scotochromogens are those cultures whose colonies are pigmented when grown in the dark. When the cultures are exposed to light continuously for 2 weeks, the pigment in the colonies may deepen to orange or red (Figure 11).

(c) Nonphotochromogens are those cultures whose pigment is not changed by exposure to light for 2 weeks.

(d) Absorbed pigment or the "greening" phenomenon may be seen when rare strains of *M. fortuitum* or *M. tuberculosis* appear to absorb the malachite green from egg medium.

(4) Colony morphology is best observed with isolated colonies. Colonies are usually described as rough, smooth, domed, etc. If the growth is confluent, then the individual colony types cannot be determined. Make a subculture onto a transparent medium. Use a small inoculum in order to have isolated colonies whose individual morphology can be seen.

For examples of rough and smooth colony types on Lowenstein-Jensen egg medium, see Figures 12-14.

Colony variations on the transparent 7H-10 agar medium may be observed with the aid of a dissecting microscope. (See Figure 15.)

Under magnification, young developing colonies are seen as clusters of bacilli that form typical colonies as they mature.

Convenient symbols for recording colony characteristics are:

Rg or R = rough
Sm = smooth
D = domed (hemispherical)
S = spreading, serrated edge
T = transparent, thin
X = xenopi
K = kansasii
"K" = resembles kansasii
f = filamentous
w = wrinkled (not rough)
I = interconvertible (a subculture of a single colony of mixed types reproduces like mixed types)
Ch = chromogenic
NC = nonchromogenic
y = yellow
o = orange
s = spot in center
a = apron (thin growth at the base of the colony)

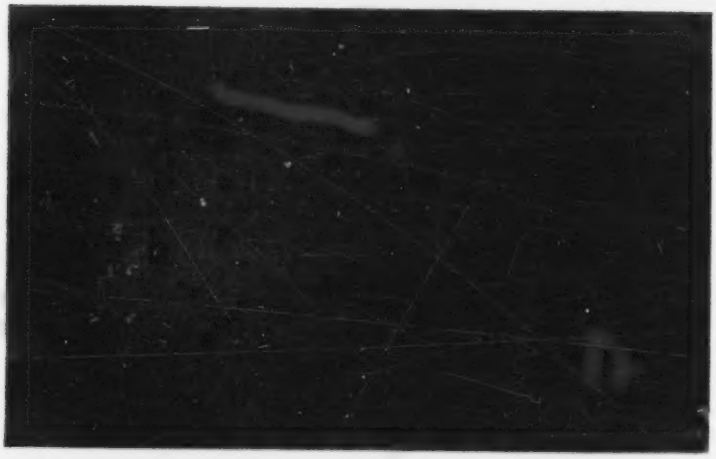
A combination of descriptive symbols may be used, such as:

RgRy = rough yellow
SmS = smooth spreading
SmSy = smooth spreading, yellow
SmDa = smooth domed with apron
SmT = smooth transparent
SmI = a subculture of a SmS or a SmD colony from a culture that has both SmS and SmD colonies produces both SmS and SmD colonies (interconvertible)

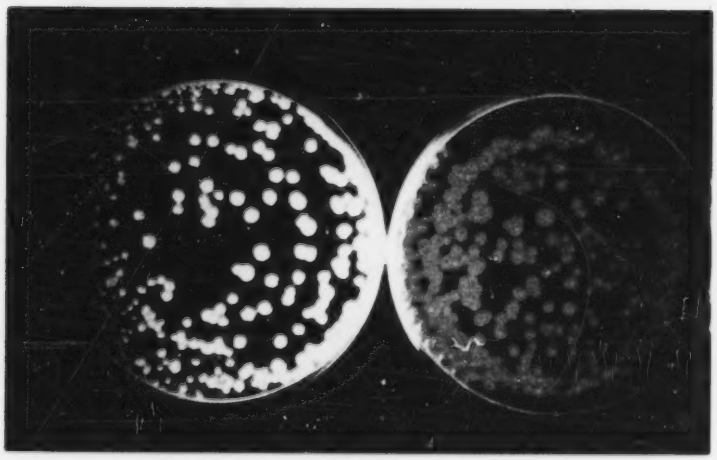
(5) When more than one colony form is seen in a culture, suspect a mixed culture or a contaminant. See Section II G. Problem Cultures.

Figure 10 — Pigment Formation in Strains of *M. kansasii* Exposed to Light

A. Rough Strain on egg medium, grown in dark



B. Same Strain after exposure to light



C. *M. kansasii* on 7H-10 plates before and after light exposure

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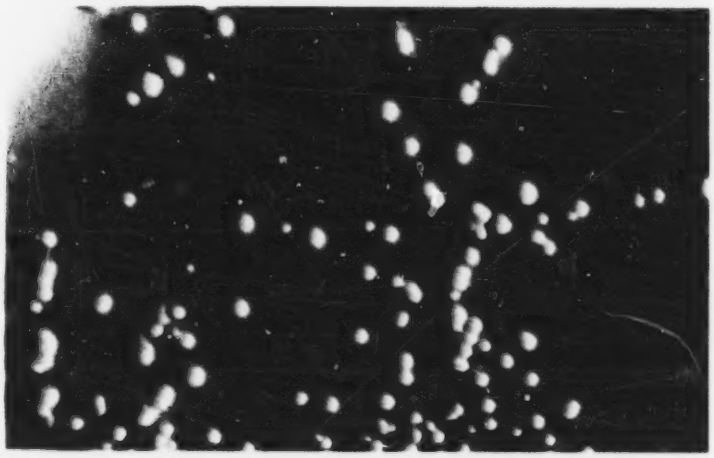
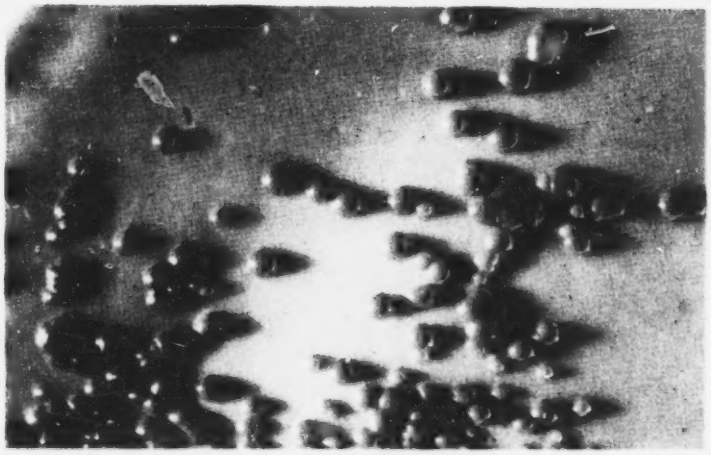
Figure 11 — Intensification of Pigment in Scotochromogen by Light Exposure



A. Grown in the dark



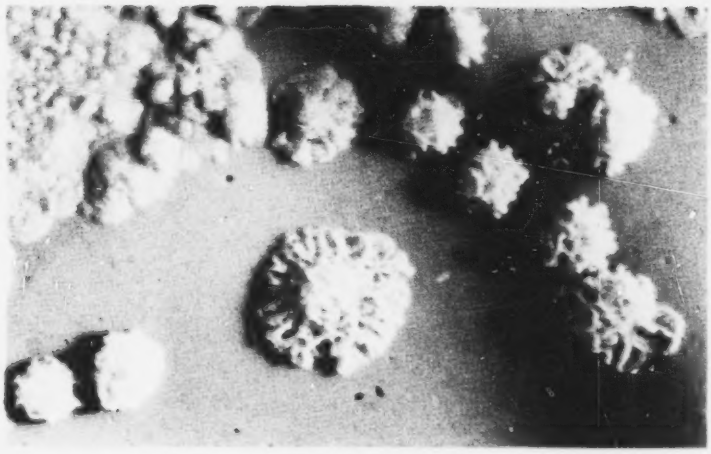
B. Exposed to light for 2 weeks

Figure 12 — Smooth Strain of *M. fortuitum* on Lowenstein-Jensen Egg Medium

Figure 13 — Examples of Rough Colony Types Observed on Lowenstein-Jensen Egg Medium

A. *M. tuberculosis*

B. *M. fortuitum*



C. *M. smegmatis*

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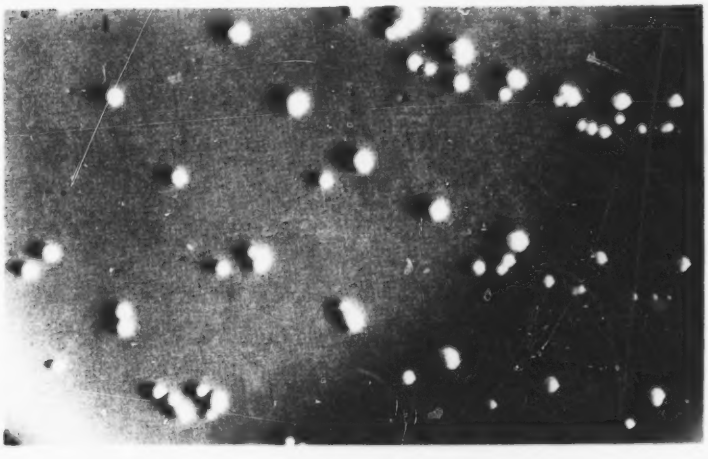
Figure 14 — Other Colony Types Seen on Lowenstein-Jensen Egg Medium

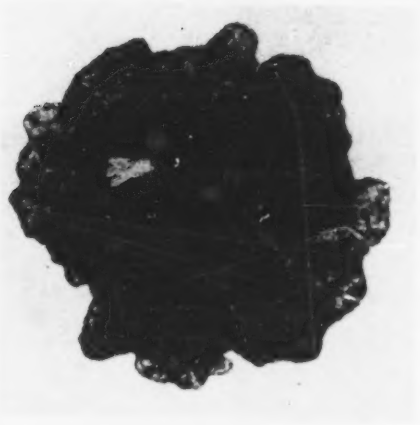
A. *M. kansasii*, rough colony

B. Close-up of translucent, pyramid-shaped colony of *M. bovis*

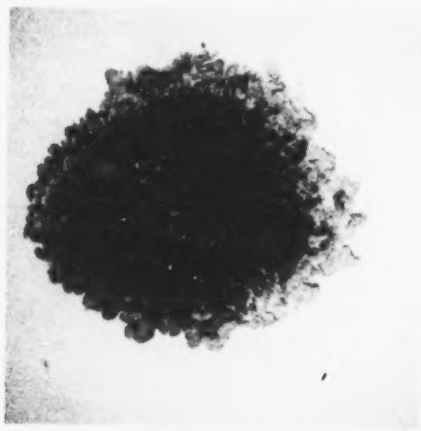


C. Nonphotochromogen (Battey)

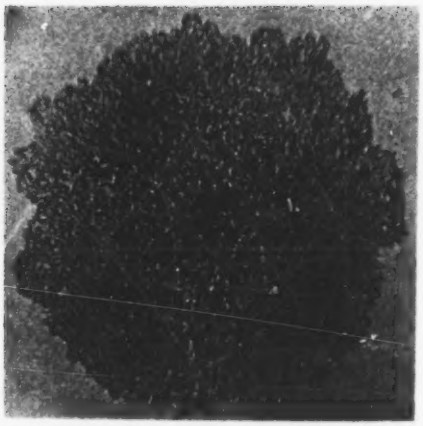
Figure 15. Colonies on 7H-10 Agar Medium



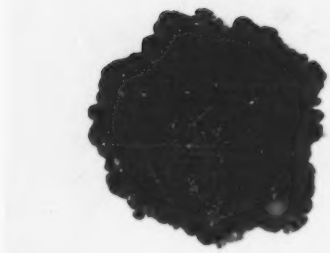
Rough R (RgR), dry, white, buff. Made up of serpentine cords with a heaped-up center. Mature in 10-20 days. Seen in *M. tuberculosis.*. *M. bovis* resembles this colony, but matures more slowly.



Rough R (RgR), white, buff, or yellow. Dense, coarse, texture. Mature in 5-7 days. Rapid growers such as *M. fortuitum, M. phlei, M. smegmatis,* others.



Rough R, white, buff, or yellow. Mature in 10-14 days. Seen in *M. kansasii,* also in scotochromogens and non-chromogens such as *M. triviale.*



Rough Rw, wrinkled as opposed to cords. Mature in 10-14 days. Seen in *M. kansasii* more often than others.

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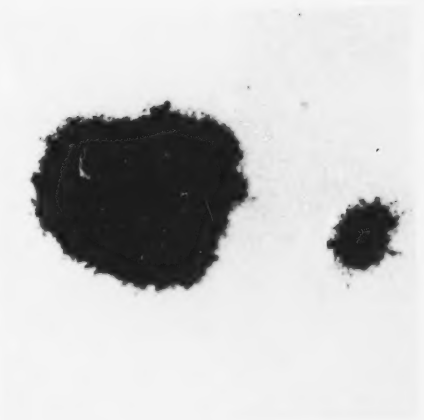
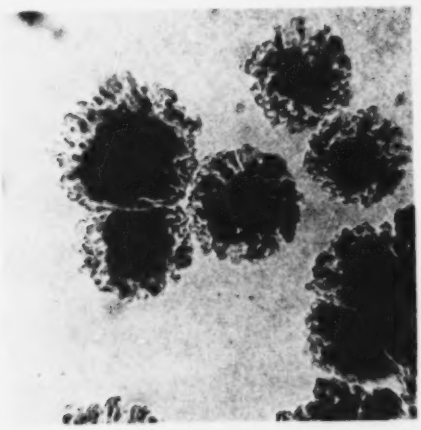
Figure 15. (Continued)

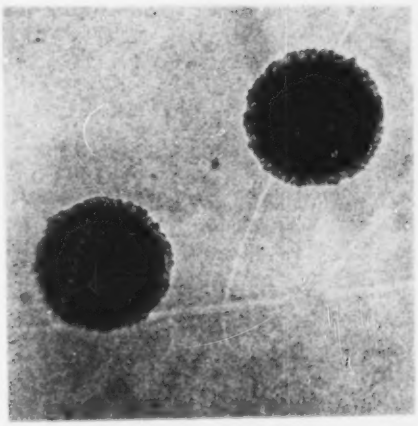


Rough X or Smooth X. Tiny domed colonies with short stick-like projections. Surface of dome may be rough or smooth. Usually yellow at maturity. Requires 21 to 28 days for minute colonies. Typical *M. xenopi*.



Smooth Kw or K. Non-chromogenic in the dark and yellow in the light. With transmitted light has a characteristic sheen. Often wrinkled as opposed to cords. Mature in 10-14 days. Typical *M. kansasii*.

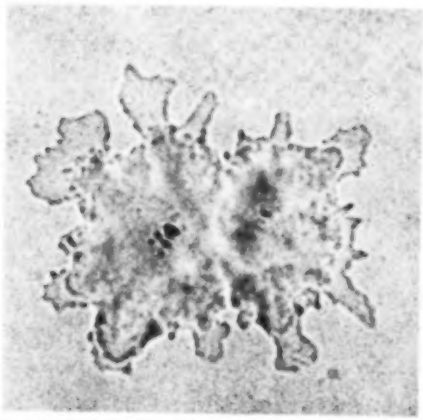
Smooth Domed (SmD). Hemispherical colonies. White, cream, yellow. Mature in 10-14 days. Seen in non-photochromogens, scotchromogens, and rapid growers. Often have granules in center.



Smooth Domed with apron of growth at base of colony (SmDa). Seen in rapid and slow growers, such as nonphotocromogens and scotochromogens. White, cream, or yellow.

Figure 15. (Continued)

Smooth Spreading (SmS). Raised center, the outer area becoming thin with an undulating edge. White, cream, yellow. Mature in 10-14 days. Seen in nonphotochromogens and scotochromogens.



Smooth transparent (SmT). Like the SmS colony, but thin with only slightly raised center. White, cream, yellow. Mature in 10-14 days. Seen in nonphotochromogens and scotchromogens.

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3. Identification Procedure.

Make a smear of the growth and stain to determine that the colonies are composed of acid-fast bacilli. This is important because contaminating organisms can interfere with the identification test results.

Separate cultures according to their growth rate and chromogenicity.

When in doubt about growth rate or chromogenicity, make a sub-culture with small inoculum to obtain isolated colonies.

Select the identification tests needed according to the level of service offered by the laboratory. (See page 15.) Never rely on one test alone to identify a species of Mycobacterium.

One quadrant or slant of medium with at least 100 colonies is a sufficient amount of growth for performing several identification tests.

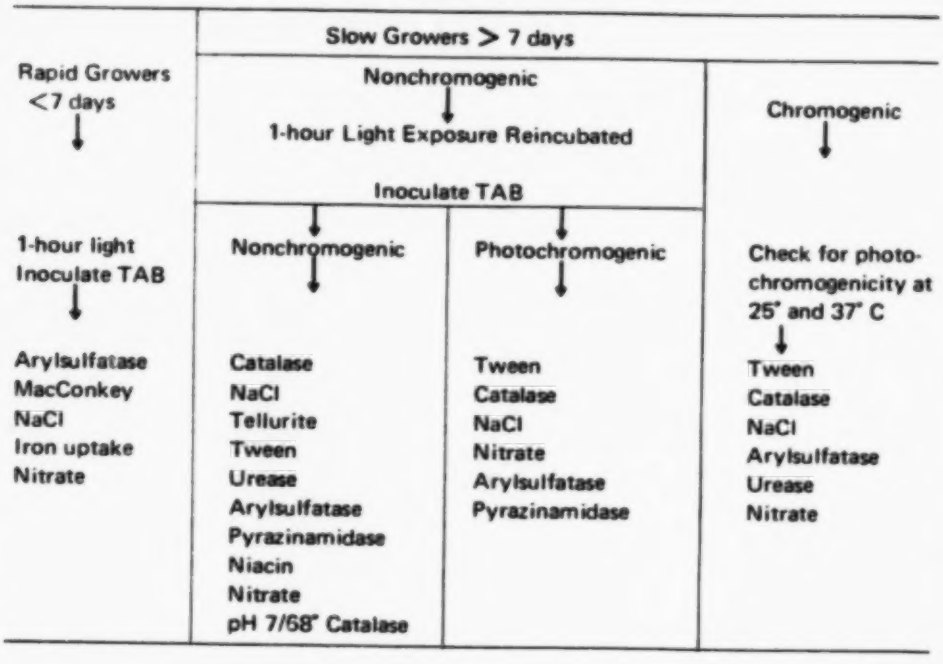
Test or inoculate as follows:

Culture	Inoculate	Page
Rapid Growers	To TAB*	
	Arylsulfatase	65
	MacConkey	74
	NaCl tolerance	84
	Iron Uptake	73
	Nitrate	78
Slow Growers (nonchromogenic and chromogenic)	To TAB	
	Tween hydrolysis	87
	NaCl tolerance	84
	Arylsulfatase	65
	Urease	89
	1-hour light (also @ 25° C if necessary)	80
	Catalase	69
	Nitrate	78
Non-chromogenic Slow growers (additional tests)	Tellurite reduction	84
	Pyrazinamidase	81
	Catalase, pH 7/68° C	71
	Niacin	75
Suspected *M. bovis*	Thiophen-2-carboxylic acid hydrazide	86
	Pyrazinamidase	81
	Rabbit (rarely needed)	83
Mixed Cultures	Pick colonies to TAB and 7H-10**	94
Insufficient Growth	To TAB and solid media	
Contaminated	Purify, then identify	93

*TAB = Tween-albumin broth, Dubos or Middlebrook 7H-9

**or other transparent agar-base medium

Work-Flow Chart for Identification of Acid-Fast Bacilli


Slow Growers > 7 days
Rapid Growers <7 days
Nonchromogenic
1-hour Light Exposure Reincubated
Inoculate TAB
Chromogenic
1-hour light Inoculate TAB
Nonchromogenic
Photochromogenic
Check for photo-chromogenicity at 25° and 37° C
Arylsulfatase MacConkey NaCl Iron uptake Nitrate
Catalase NaCl Tellurite Tween Urease Arylsulfatase Pyrazinamidase Niacin Nitrate pH 7/68° Catalase
Tween Catalase NaCl Nitrate Arylsulfatase Pyrazinamidase
Tween Catalase NaCl Arylsulfatase Urease Nitrate

To identify a culture, consult the "Key Characteristics," Tables II, III, IV and V. Table VI summarizes the usual reactions of all the species to all of the tests, regardless of their usefulness for identification.

When results of identification tests do not conform to the usual results for classifying an organism, repeat the test or tests at least once. Variations may be due to (1) contamination, (2) mixed cultures, (3) biological variation, and/or (4) environmental influences such as CO_2, O_2, media, moisture, etc.

If the tests have been repeated and the organism still does not fit a known species pattern, refer the culture to a specialty laboratory, e.g., a state health department laboratory or the CDC.

4. Species Identification

a. RAPID GROWERS

Mature colonies are visible without magnification in less than 7 days. *M. fortuitum* and *M. chelonei* are the potential pathogens in this group.

Because both *M. chelonei* and *M. fortuitum* can produce pathology in man, there should be no reason for concern if they are not properly speciated. In fact when only 3-day arylsulfatase and MacConkey agar tests are done,

the report of *M. fortuitum* complex may be sufficient for the clinician.

Other species such as *M. vaccae*, *M. smegmatis*, and *M. phlei* are considered saphrophytes and require special tests for taxonomic identification.

(1) Colony characteristics

On Lowenstein-Jensen or 7H-10 medium the colonies may be rough or smooth.

On 7H-10 the Rough forms seem to be composed of coarse cords; the smooth forms are very opaque and dense but slope off near the periphery of the colony, allowing light to penetrate the irregular outer edge. Other rapid growers produce a variety of opaque, patterned colony forms.

(2) Pigment production

M. fortuitum and *M. chelonei* are nonchromogenic. Most other rapid growers are nonchromogenic; however, a few may be yellow, orange, or pink. The pigment of *M. vaccae* is very susceptible to light and may change to bright yellow after only a few minutes of exposure. If freshly inoculated cultures of this organism are immediately covered to prevent light exposure, they usually grow without pigment.

(3) "Greening" on egg medium

M. fortuitum colonies may appear to absorb malachite green.

Table II
Key Characteristics of Rapid Growers

Species	Pigment	Nitrate Reduction	5% NaCl Growth	Arylsulfatase* 3 day	Arylsulfatase* 2 wk	Mac-Conkey	Iron Uptake
M. fortuitum	NC	+	+	±/2+	3/5+	+	+
M. chelonei	NC	–	V	±/2+	3/5+	+	–
M. smegmatis	NC	+	+	–	–/3+	–	+
M. phlei	y	+	+	–	–/3+	–	+
M. vaccae	y**	+	+	–	1/3+	–	+
"Others"	V***	+	V	–/1+	–/5+	–	+

*When results are separated by a slash, as in ±/2+, variations in test reactions, low to high are indicated.
**Photochromogenic
***V = variable

b. **NONPHOTOCHROMOGENS (SLOW GROWERS)**

Mature colonies require more than 7 days to be visible without magnification. Pigmentation varies from white to buff, tan, or pale yellow and does not intensify upon light exposure.

(1) *M. tuberculosis* is the species in this group most commonly isolated from pulmonary disease. Colonies are characteristically rough and appear to be formed by serpentine growth that piles up in the center. They are nonchromogenic (cream to buff color), grow at 35°-37° C, and usually require 14 days or longer to produce macroscopically visible colonies. Mature cultures are niacin positive, nitrate reductase positive, and their catalase activity is inactivated at pH 7 when heated at 68°C for 20 minutes. Test results for Tween hydrolysis and tellurite reduction are variable and of no value in identifying *M. tuberculosis*; however, these tests should be set up because they are necessary for the identification of niacin negative, nonchromogenic slow growers.

Drug resistant strains of *M. tuberculosis* may have granular, almost smooth colonies on Lowenstein-Jensen medium; on 7H-10 they are consistently rough.

(2) *M. bovis* rarely occurs in man in the U.S.A., but it may be isolated from clinical specimens. Colonies on Lowenstein are tiny, smooth, pyramidal, and translucent, often resembling the surface of the medium. On 7H-10 the colonies are always rough, resembling *M. tuberculosis*. This species, the least reactive in the tests, gives a positive result only in the room temperature and the semi-quantitative catalase tests. *M. bovis* is usually niacin negative but an occasional strain can be weakly niacin positive. The pyrazinamidase test and sensitivity to thiophen-2-carboxylic acid hydrazide (TCH) aid in separating it from *M. tuberculosis*. Confirmation by rabbit inoculation is rarely required.

(3) *M. avium* complex causes disease in fowl, swine, and occasionally in other animals, but rarely in man. The closely related organism, *M. intracellulare*, is known to cause human disease, especially in Southeastern

United States. Because a recent international cooperative study revealed the close taxonomic similarity of *M. intracellulare* to *M. avium*, it is acceptable to lump these two species together under the designation of *M. avium* complex. However, the name *M. intracellulare* may continue to be used by some investigators.

M. avium complex cultures usually have predominately Smooth S, T, or D colony forms on primary isolation. Occasionally rough strains are seen. More than one colony form may be found within the same culture. Colonies sometimes show varying shades of pale pigment as they become older; when yellowish, they may sometimes be confused with the scotochromogen, *M. scrofulaceum*. They are niacin negative, nitrate negative, Tween hydrolysis negative at 10 days, tellurite reduced in 3 days, urease negative, and pyrazinamidase positive.

(4) *M. xenopi* may be implicated in human disease. Its optimum growth temperature is 42° C, but it will grow at 35°-37°C, and will produce tiny visible colonies in 4-6 weeks. With magnification, the colony on 7H-10 medium resembles a miniature "bird's nest" with a rough or smooth dome having short stick-like projections visible at the periphery. These unique colonies have been labeled Rough X and Smooth X. Mature colonies are yellow. They are nitrate negative, Tween hydrolysis negative, and urease negative.

(5) *M. kansasii* is nonpigmented when grown in the dark. It characteristically has a Smooth K colony on 7H-10; a few rough strains may be encountered and mistyped as *M. tuberculosis* or another rough nonpigmented Mycobacterium unless the light exposure test is properly performed. For a description of the species, *M. kansasii*, see Photochromogens, page 57; however the characteristics of the species are also included in Table III.

(6) *M. ulcerans* may be isolated from ulcerative cutaneous or sub-cutaneous lesions but should never be expected in sputum or gastric lavage. It grows very slowly, requiring 6-9 weeks at 30°-33° C for the rough colonies to appear. It does not grow at 37° C.

It is usually niacin negative, and nitrate is not reduced. *M. ulcerans* is more common in tropical areas than in the temperate zones. *M. marinum* also causes cutaneous lesions, but it is a photochromogen.

(7) The nonphotochromogens which are not considered clinically significant are *M. terrae* complex, *M. gastri,* and *M. triviale.*

M. terrae complex: colonies may be rough or smooth. This complex includes the closely related organisms *M. nonchromogenicum, M. terrae,* and possibly others as yet not identified. These organisms are commonly found in the soil and water, and subsequently in clinical specimens.

M. gastri: the colonies resemble Smooth "K" on 7H-10 except the central spot is smaller. These have been isolated from gastric washings.

M. triviale: This species produces thin rough colonies on 7H-10 and usually rough colonies on Lowenstein. They may be isolated from clinical specimens.

See Table III, Key Characteristics, page 58.

c. PHOTOCHROMOGENS (SLOW GROWERS)

The unique characteristic of the photochromogens is that the colonies are nonpigmented when grown in the dark, but when exposed to light for 1 hour and then reincubated for 6-24 hours, they become chromogenic.

Cultures with confluent growth or old colonies may not produce pigment after exposure to light for 1 hour, and a subculture may be necessary. Careful light studies and observation of colony characteristics are important because identification test results are similar to those obtained with clinically insignificant mycobacteria.

(1) *M. kansasii* colonies on 7H-10 are Smooth K, or Kw. Rough strains also occur. Exposure to continuous light for 2 weeks during incubation often produces orange crystals of carotene within isolated colonies. Strains of *M. kansasii* with high catalase activity have been associated with disease more often than those strains with low catalase activity.

(2) *M. marinum* may be isolated from superficial lesions and is never to be expected in sputum. Its colony

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Table III

Key Characteristics of Nonphotochromogens

Species	Colony Characteristics 7H-10	Colony Characteristics L-J	1 hr. Light	Niacin	Nitrate Reduction	Catalase Rm* Temp	Catalase SQ mm	Catalase pH7 68°	Tween Hydrol. (days)	Tellurite Reduction 3 days	Growth on 5% NaCl	Arylsulfatase 3 da	Arylsulfatase 2 wk	TCH sens.	Pyrazinamidase 4-da	Pyrazinamidase 7-da	Urease
POTENTIAL PATHOGENS																	
M. tuberculosis	Rg	Rg	–	+	3/5+	S	<45	–	>5	–	–	–	–	–	+		+
M. bovis	Rg	Sm	–	–	–	S	< 45	–	>5	–	–	–	–	+	–	–	+
M. kansasii	K or Rg	Sm or Rg	+	–	3/5+	R	>45	+	<5	–	–	–	–/1+	–	–	+	+
M. xenopi	SmX-RgX	Sm	–	–	–	S	<45	+	–	–	–	±	2/5+	–	+		–
M. avium complex**	SmI-Rg	Sm or Rg	–	–	–	S	<45	+	–	+	–	–	–/2+	–	+		–
M. ulcerans	Rg	Rg	–	–	–	R	>45	+	–			–		–			
NON-PATHOGENS																	
M. terrae complex	SmI-Rg	Sm or Rg	–	–	±/4+	R	>45	+	5-10	–	–	–	–/2+	–	–/+	+	–
M. gastri	"K" like	Sm	–	–	–	S	<45	–	5-10	–	–	–	–	–	–	–/+	+
M. triviale	Rg	Rg	–	–	3/5+	R	>45	+	5-10	–	+	±	3/5+	–	–/+	+	–

* Reaction R = rapid, S = slow

** *M. avium* complex includes *M. intracellulare*

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morphology is similar to that of *M. kansasii.* Growth is more rapid at 30°-32° C than at 37° C, or it may not grow at 37° C; therefore isolates from skin lesions must be incubated at the lower temperature to assure growth.

(3) *M. simiae (habana)* is known to produce disease in man and has been identified from clinical material. It is a niacin positive photochromogen. Few other *in vitro* properties have been documented.

Table IV
Key Characteristics of Photochromogens

Species	Colonies on 7H-10	Colonies on L-J	Niacin	Nitrate Reduction	Catalase >45	Catalase 68°	Arylsulfatase 3 da.	Arylsulfatase 2 wk.	Pyrazinamidase* 4 da.
M. kansasii	K or Rg	Sm or Rg	—	+	+/—	+	—	—/1+	—
M. marinum	"K"	Sm	—/+	—	+	—/+	—/2+	4+	+
M. simiae (habana)	Sm	Sm	+	—/+	+	+	?	?	?

*Perform only when other tests indicate *M. marinum* or send culture to a reference laboratory.

d. SCOTOCHROMOGENS (SLOW GROWERS)

The potential pathogens, *M. scrofulaceum* and *M. szulgai,* should be identified. *M. gordonae* and *M. flavescens* are considered nonpathogens. *M. flavescens* is intermediate in speed of growth and may be considered either a rapid or slow grower.

(1) Colony characteristics

Smooth with domed or spreading colonies having a definite yellow pigment even when grown in the dark. The scotochromogens have a wide variety of colony forms: Smooth Sy, Dy, Ty, or an occasional Rough Ry.

(2) Effect of light on pigment

When exposed to "continuous" light for 2 weeks, many strains increase in pigment from yellow to orange or brick red. If cultures are exposed to continuous light, they should be incubated in the dark until visible colonies appear and then placed in

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continuous light. Initial growth may be inhibited by too much light.

Chromogenic cultures that have been exposed to light in the incubator or intermittently during weekly culture reading should be subcultured for the light exposure test to avoid an incorrect identification of *M. kansasii.*

(3) *M. szulgai* may be photochromogenic when grown at 25°C; at 37° C it is scotochromogenic.

(4) The pale yellow *M. xenopi* has been described along with the nonchromogenic slow growers because of its biochemical similarity to the *M. avium* complex.

(5) *M. scrofulaceum* is urease positive. *M. gordonae,* the yellow strains of the *M. avium* complex, and *M. xenopi* are urease negative.

Table V
Key Characteristics of Scotochromogens

Species	Pigment			Nitrate Reduction	Tween Hydrol. (days)	5% NaCl Growth	Arylsulfatase		Urease
	dark	light	1 hr exp				3 da	2 wk	
M. scrofulaceum	y	y-o	–	–	–	–	–	–	+
M. gordonae	y	y-o	–	–	5-10	–	–	±	–
M. szulgai	y	y-o	–*	+	>5	–	–/+	+	+
M. flavescens	y	y-o	–	+	5-10	±	–	–/4+	+
M. kansasii	NC	y-o	y	+	<5	–	–	–/1+	+

* 37 C incubation. At 25°C *M. szulgai* may be photochromogenic.

5. Unusual or Rarely Encountered Species

Four of the species listed in Table I of this section are identified only rarely in the United States. Reasons for their rarity are varied, but include the following: a) newly described species, only a few strains of which have been studied; b) uncertainty about taxonomic features due to limited number of strains studied; and c) sharply demarcated geographic distribution. Increasing global travel to foreign countries, as well as the influx of immigrants, may result in the occasional isolation in the United States of species not commonly

encountered in this country. Because of this, data thus far accumulated on these four species is presented here so that laboratories will be aware of at least some of their differential features.

M. africanum appears to be limited to central and western Africa, where it causes tuberculosis in man. Suspect strains have been isolated from African monkeys brought into the United States. The organism is taxonomically variable and intermediate between *M. tuberculosis* and *M. bovis*, which suggests either geographic variation in properties or the existence of truly distinct subspecies of the *africanum* organisms in different parts of Africa. *M. africanum* grows very slowly, not uncommonly requiring more than 42 days for appearance of visible growth. Colonies usually are dysgonic (similar to *M. bovis*), but growth is greatly enhanced by the addition of 0.1% to 0.2% pyruvate to the primary isolation medium. Niacin test results with the organism vary; most strains are negative, but a significant number yield positive reactions. Although results with the nitrate reduction test are commonly negative, some positive reactors have been noted. The organisms commonly are susceptible to TCH and pyrazinamide, while resistance to amithiazone (TB-1 or conteben) varies and appears to be related to geographic area. Although *M. africanum* is regarded as a distinct, easily defined entity in Africa, the limited few strains seen in this country make this species a taxonomic puzzle.

M. simiae was first described in monkeys; however, recent comparative studies suggest it is the same organism as *M. habana*, which has been isolated from humans with pulmonary disease in Cuba. By international rules of nomenclature, the name *M. simiae* has priority over *M. habana*, which should be regarded as a subjective synonym. Although still a rarely encountered mycobacterium in this country, *M. simiae* might become more important with the immigration of people from those parts of the two American continents which lie south of the United States. Few properties have been documented for *M. simiae*; however, it is reported to be strongly niacin positive, photochromogenic, nitrate reductase negative, strongly catalase positive, positive 68° catalase, and negative in Tween hydrolysis.

Little information is available concerning *M. szulgai*; however, all strains thus far encountered have been implicated as human pathogens. The limited few strains examined indicate that *M.*

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szulgai is usually slowly positive in Tween hydrolysis (> 7 days), positive in nitrate reduction, commonly scotochromogenic at 37°C but may be photochromogenic when grown at 25°C, urease positive, and arylsulfatase positive at 2 weeks, with some strains positive in 3 days.

M. ulcerans, the causative agent of ulcerative cutaneous or subcutaneous lesions, has never been isolated from sputum or gastric washings. Commonly localized in tropical and sub-tropical climates, *M. ulcerans* is rarely, if ever, seen in the United States or other temperate zones. This organism grows very slowly, taking 6-9 weeks on primary isolation, and grows only at 30°-33°C (will not grow at 37°). *M. ulcerans* is commonly negative in niacin, nitrate reduction, and Tween hydrolysis tests, but produces >45 mm of bubbles in the semiquantitative catalase test, and is positive in the 68°C catalase test.

From the foregoing it is obvious that in the United States current knowledge of and experience with the above 4 species of mycobacteria is limited. For this reason, any isolate thought or suspected to be one of these taxa should be sent for confirmation to a large reference laboratory (e.g., one of the Veterans Administration Mycobacteriology Reference Laboratories or the Mycobacteriology Branch, Center for Disease Control).

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TABLE VI

SUMMARY TABLE: GROWTH CHARACTERISTICS AND IDENTIFICATION OF MYCOBACTERIA *

Variations are to be expected.

Species	Growth in less than 7 days	Pigment Formation			Niacin	Nitrate Reduction	Catalase Activity			Tween Hydrolysis (days)	Tellurite Reduction in 3 days	Growth on 5% NaCl	Arylsulfatase		Growth on MacConkey Agar	Clinical Significance
		Dark	Light	1 hr			Rm Temp	SQ mm	pH7 68°				3-da	2-wk		
M. tuberculosis	-	-	-	-	+	3/5+	S	<45	-	>5	-	-	-	-	-	+
M. bovis	-	-	-	-	-	-	S	<45	-	>5	-	-	-	-	-	+
M. kansasii	-	-	+	+	-	3/5+	R	>45	+	<5	-	-	-	-/1+	-	+
M. marinum	±	-	+	+	V	-	S	<45	±	<5	-	-	-/2+	4+	-	+
M. simiae	-	-	+	+	+	-/+	R	>45	+	>5						+
M. scrofulaceum	-	+	+	-	-	-	R	>45	+	-	-	-	-	-	-	+
M. gordonae	-	+	+	-	-	-	R	>45	+	5-10	-	-	-	±	-	-
M. szulgai	-	±	+	**	-	+	R	>45	+	>5	-	-	-/+	+	-	+
M. flavescens	±	+	+	-	-	+	R	>45	+	5-10	-	±	-	-/4+	-	-
M. avium complex***	-	-	-	-	-	-	S	<45	+	-	±	-	-	-/1+	±	+
M. ulcerans	-	-	-	-	-	-	R	>45	+	-			-			+
M. xenopi	-	+	+	-	-	-	S	<45	+	-	-	-	±	2/5+	-	+
M. gastri	-	-	-	-	-	-	S	<45	-	5-10	-	-	-	-	-	-
M. terrae complex	-	-	-	-	-	1/5+	R	>45	+	5-10	-	-	-	-/2+	-	-
M. triviale	-	-	-	-	-	1/5+	R	>45	+	5-10	-	+	±	3/5+	-	-
M. fortuitum	+	-	-	-	-	3/5+	R	>45	+	±	V	+	+/2+	3/5+	+	+
M. chelonei	+	-	-	-	V	-	R	>45	+	-	V	V	2/3+	4/5+	+	+
M. smegmatis	+	-	-	-	-	1/3+	R	>45	+	<5	+	+	-	3+	-	-
M. phlei	+	+	+	-	-	1/3+	R	>45	+	<5	+	+	-	-/3+	-	-
M. vaccae	+	+	+	+	-	1/3+	R	>45	+	<5	V	+	-	3+	-	-

* See Key Tables II-V for additional differential tests.

** M. szulgai may be photochromogenic when grown at 25° C.

*** M. avium complex includes M. intracellulare.

Key to symbols:

- = usually negative
+ = negative, rarely positive
± = positive, rarely negative
+ = usually positive
/ = low to high variations

V = variable
R = rapid
S = slow
Space = lack of information.

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SECTION II F. IDENTIFICATION TEST TECHNIQUES

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*Tests not routinely performed at CDC

F. IDENTIFICATION TEST TECHNIQUES

Most mycobacteria may be identified by rate of growth, pigment and colonial characteristics, together with two or more other properties. Specific identification of an isolate should never be based upon a single test or characteristic because individual strains may deviate from the expected results of a given taxon. On the basis of initial observations on pigment production and speed of growth, it is possible to select those tests which will prove most valuable in establishing the identity of the strain in question. To save time and insure efficiency in test performance and interpretation, it is recommended that, with rare exceptions, all isolates to be identified be subjected to all *in vitro* tests here described. For final identification, however, only those selected key tests described in Section IIE need be considered for the species listed.

Methods for performance and interpretation of key tests that have been found valuable for identification of clinically significant mycobacteria are detailed in this section.

1. Arylsulfatase

The two methods most commonly employed to detect this enzyme utilize liquid substrate medium (CDC methods) or solid medium (Wayne modification).

CDC Methods

There are two tests: a 3-day test and a 2-week test; the former is most valuable in differential identification of rapidly growing mycobacteria and some few slow growers, while the latter is used primarily with slowly growing species.

a. CULTURES

Inoculate all mycobacteria to substrate media for both the 3-day test and the 2-week test. Potentially pathogenic rapid growers commonly are positive in 3 days, while normally saphrophytic species are not positive until 2 weeks. Those slow growers for which the 3-day test and/or the 2-week test may be useful include *M. marinum, M. szulgai, M. xenopi, M. triviale* and *M. flavescens.*

b. LIQUID SUBSTRATE MEDIUM

Prepare stock substrate

(1) Dissolve 2.6 g phenolphthalein disulfate, tripotassium salt, in 50 ml distilled water; this is an 0.08 M solution.

(2) Sterilize by membrane filtration.

(3) Store in refrigerator.

NOTE: *Before use, the phenolphthalein disulfate should be tested for free phenolphthalein. Dissolve a small portion in a minimal amount of water and add a few drops of 2N sodium carbonate or other alkali. If a pink color forms, this indicates free phenolphthalein, which will give false positive reactions under actual test conditions. Sufficient purification of product may be attained by dissolving 3 g to 5 g of phenolphthalein disulfate in a minimal amount of water; this salt may be precipitated from solution (free of most phenolphthalein) by the addition of excess ethanol. The precipitated disulfate salt may be filtered off on a Buchner funnel, washed several times with fresh ethanol, allowed to dry, and bottled for use in preparation of stock solution as described above.*

Prepare Liquid Medium

(1) Use either Dubos broth or Middlebrook 7H-9.

(2) Prepare two flasks, each with 200 ml of the sterile liquid medium.

Final Substrate Medium

(1) Add 2.5 ml of the 0.08M stock substrate to 200 ml of sterile liquid medium. This provides 0.001M substrate for the 3-day test.

(2) Add 7.5 ml of the 0.08M stock substrate to 200 ml of sterile liquid medium. This provides 0.003M substrate for the 2-week test.

(3) Aseptically dispense each substrate in 2 ml amounts into sterile 16 x 125 mm screw-cap test tubes.

(4) The ready-to-use substrates may be stored at room temperature. As a quality control check, add to a tube of each uninoculated substrate a few drops of sodium carbonate. The substrates should remain colorless. If solution turns pink, discard the substrate and check quality of stock powder (as

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indicated in NOTE above) before preparing fresh substrate.

c. **REAGENT**

2 N sodium carbonate (10.6 g anhydrous Na_2CO_3 in 100 ml H_2O)

d. **CONTROLS**

A tube of uninoculated substrate medium (negative).
A tube of each substrate inoculated with *M. fortuitum* (positive).

NOTE: *Prepare a color standard for reading the aryl-sulfatase test results; see Appendix C.*

e. **PROCEDURE**

(1) Label tubes of substrate with specimen numbers.

(2) Inoculate the tubes with 0.1 ml of a 7-day liquid culture of the organism to be tested *or* a spadeful of organisms from a freshly grown culture.

(3) Incubate at 37°C.

(4) After 3 days of incubation of the 0.001 M substrate, remove tests from the incubator and add not more than 6 drops of 2N sodium carbonate solution (see f. Results).

(5) After 2 weeks of incubation of the 0.003 M substrate, remove tests from the incubator and add 2N sodium carbonate as in (4).

f. **RESULTS**

Positive = a color change to pink or red.
Negative = no color change.

Compare results with a set of color standards and record intensity of color (- to 5+).

References:

Kubica, G.P. and Ridgon, A.L. 1961. The arylsulfatase activity of acid-fast bacilli. III. Preliminary investigation of rapidly growing acid-fast bacilli. Amer. Rev. Resp. Dis. 83: 737-740.

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Whitehead, J.E.M., Wildy, R. and Engbaek, H.C. 1953. Arylsufatase activity of mycobacteria. J. Path. Bact. 65: 451-460.

Wayne Modification

a. **CULTURES**

Inoculate all mycobacteria to substrate medium. Unlike the CDC methods, this procedure utilizes an unenriched agar base medium; hence most consistent results are obtained only for the 3-day test, and commonly only with the rapidly growing mycobacteria.

b. **SUBSTRATE PREPARATION***

To 100 ml of melted Dubos oleic-agar base, add 1 ml of glycerol and 65 mg of tripotassium phenolphthalein disulfate. Dispense in 2 ml amounts to 18 x 160 mm flat bottomed screw-cap vials. Autoclave. Permit to harden in an upright position.

c. **REAGENT**

2 N sodium carbonate (10.6 g anhydrous Na_2CO_3 brought to 100 ml with distilled water).

d. **CONTROLS**

A tube of uninoculated substrate (negative).
A tube of substrate inoculated with *M. fortuitum* (positive).

e. **PROCEDURE**

(1) Prepare a barely turbid suspension of bacilli in sterile water.

(2) Inoculate 1 drop of this suspension to the substrate.

(3) Incubate at 35°-37° C for 3 days.

(4) Remove from incubator and add 1 ml of the Na_2CO_3 solution to the growth on the substrate.

f. **RESULTS**

Positive = development of pink to red color on the surface.
Negative = no color change.

Reference:

Wayne, L.G. 1961. Recognition of *Mycobacterium fortuitum* by means of the 3-day phenolphthalein sulfatase test. Amer. J. Clin. Pathol. 36: 185-197.

*Wayne's sulfatase medium is available commercially in powder form.

2. Catalase

Virtually all mycobacteria, except for certain isoniazid-resistant mutants of *M. tuberculosis* and *M. gastri*, possess catalase enzymes. Three different tests for catalase activity in common use are:

Room Temperature or Drop Method
pH 7/68°C test
Semiquantitative Test

a. CULTURES

As soon as colony forms are mature, cultures should be tested for catalase activity.

b. EQUIPMENT

Pipettes: 5 ml, 1 ml, or capillary.
Screw-cap test tubes, 16 x 125 mm.
Water bath or constant temperature block heater for 68°C test.
Inoculating spade or loop.

c. REAGENTS

(1) 30% hydrogen peroxide (Superoxol).
Store in refrigerator.

(2) 10% Tween 80 prepared as follows:
Mix 10 ml Tween 80 with 90 ml of distilled water. Autoclave for 10 minutes at 121°C. If Tween settles during autoclaving, swirl to resuspend. Store in the refrigerator.

(3) Immediately before performing the test, mix equal parts of Tween solution and Superoxol. (Approximately 0.5 to 1.0 ml of the mixture will be used for each test.)

(4) M/15 phosphate buffer for the pH 7/68°C test.

d. CONTROLS

Uninoculated pH 7 buffer (negative).
Uninoculated medium (negative).
M. fortuitum culture (positive).

e. PROCEDURES

Room Temperature (R.T.) or Drop Method

(1) Add 1-2 drops of the Tween-peroxide mixture to the culture growth on the plate or slant.

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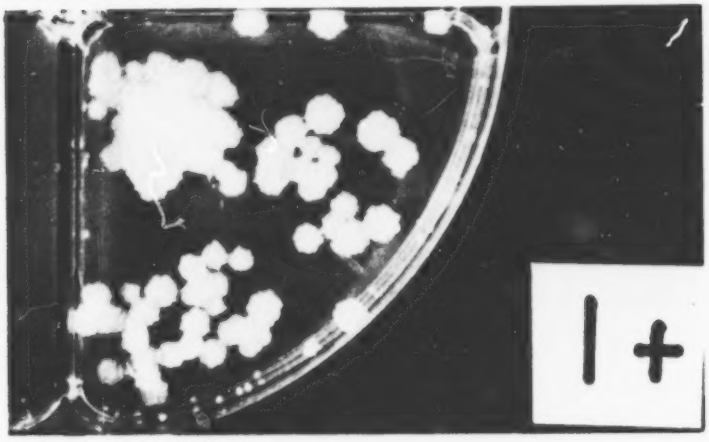
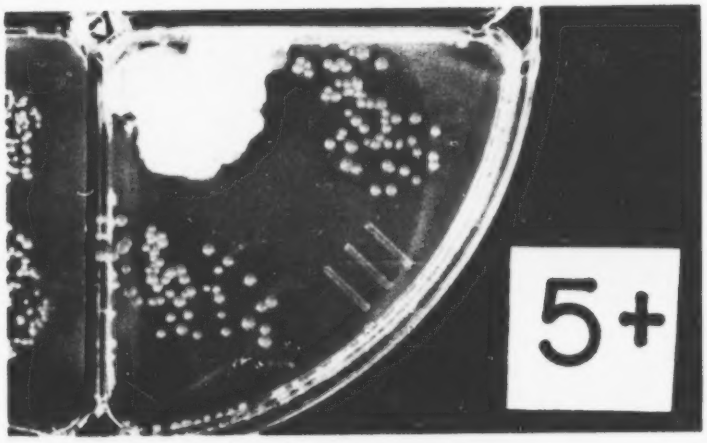
(2) Observe for the formation of bubbles. This may require 5 minutes.

(3) Results
Positive (rapid) = immediate profusion of bubbles.
Positive (slow) = few slowly forming bubbles.
Negative = no bubbling.
For examples, see Figure 16.

Figure 16. Catalase Activity Observed on 7H-10 Agar Medium



A. A 1+ reaction with bubbling observed near the center of the dish (Upper left portion of picture)



B. Violent "marshmallowy" bubbling (5+)

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pH7/68°C catalase

Certain mycobacteria lose catalase activity when suspended in pH 7 buffer and heated to 68°C; these include *M. tuberculosis, M. bovis, M. gastri* and some strains of *M. marinum* and *M. avium* complex. All non-chromogenic slow growers should be subjected to this test.

(1) Number screw-cap tubes to correspond to cultures to be tested.

(2) With a sterile pipette, add 0.5 ml phosphate buffer, pH 7, to each tube.

(3) With a sterile spade or loop emulsify several colonies from the culture into the buffer.

(4) Place the tubes containing emulsified colonies in a water bath or constant temperature block heater at 68°C for 20 minutes.

Time and temperature are important.

(5) Remove from heat and cool to room temperature.

(6) Add to each tube 0.5 ml of the Tween-peroxide mixture.

(7) Observe for formation of bubbles appearing on the surface of the liquid. Hold tubes for 20 minutes before discarding as negative.

(8) Results

Positive = bubbles.
Negative = no bubbles.

Take care not to shake tubes because Tween 80 alone may form bubbles when shaken and give a false positive reaction.

R.T. and pH 7 tests can be performed on the same slant or plate of the culture. Scrape colonies for pH 7 before testing at R.T.

References:

Kubica, G.P., Jones, W.D. Jr., Abbott, V.D., Beam, R.E., Kilburn, J.O. and Cater, J.C. Jr. 1966. Differential identification of mycobacteria. I. Tests on catalase activity. Amer. Rev. Resp. Dis. 94: 400-405.

Kubica, G.P. and Pool, G.L. 1960. Studies on the catalase activity of acid-fast bacilli. I. An attempt to subgroup these organisms on the basis of their catalase activities at different temperatures and pH. Amer. Rev. Resp. Dis. 81: 387-391.

Semiquantitative Catalase*

This test provides more standardized results than the room temperature test, and thereby enables a more definitive taxonomy of the mycobacteria under test.

Preparation of Test Cultures

(1) Prepare special tubes of medium by dispensing 5 ml of liquid Lowenstein medium into sterile 20 by 150 mm screw-cap test tubes.

(2) Inspissate for 50 minutes with tubes in an upright position to form a butt of solid medium. This may be done in a water bath adjusted to 85°C, if the usual inspissator is not suitable.

(3) Inoculate surface of butt medium with 0.1 ml or a 3 mm loopful of a 7-day-old liquid culture of the test organism.

(4) Incubate at 37°C with *caps loose* for *2 weeks.*

Procedure

(1) To the 2-week old culture, add 1.0 ml of Tween-peroxide mixture.

(2) Leave culture in an upright position at room temperature for 5 minutes.

(3) Measure in millimeters the height of the column of bubbles above the surface of the medium.

NOTE: *A convenient reading guide may be devised by drawing lines at 10 mm intervals across a 3" x 5" card.*

(4) Results

Weak positive (<45) = column of bubbles less than 45 mm.
Strong positive (>45) = column of bubbles greater than 45 mm.
Negative = no bubbling.
For examples, see Figure 17.

*Lowenstein butt-tubes are available commercially.

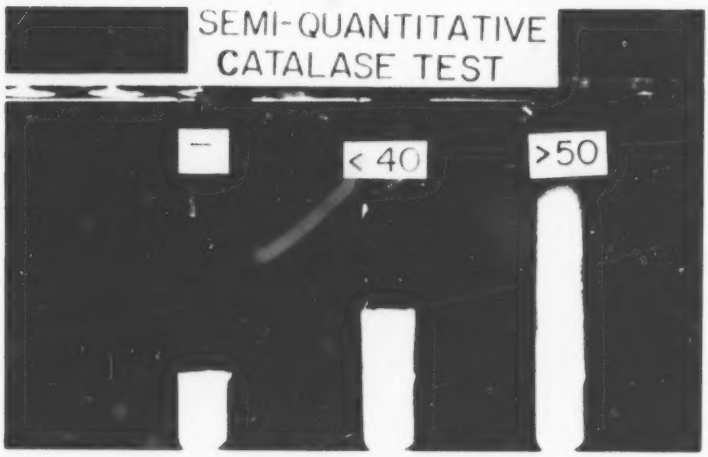

Figure 17. Semiquantitative Catalase Test

References:

Kubica, G.P., Jones, W.D. Jr., Abbott, V.D., Beam, R.E., Kilburn, J.O. and Cater, J.C., Jr. 1966. Differential identification of mycobacteria. I. Tests on catalase activity. Amer. Rev. Resp. Dis. 94: 400-405.

Wayne, L.G. 1962. Two varieties of *Mycobacterium kansasii* with different clinical significance. Amer. Rev. Resp. Dis. 86: 651-656.

3. Iron Uptake

a. CULTURES

In the iron uptake test, *M. fortuitum* is positive and *M. chelonei* is negative. Most other rapid growers are positive. Slow growers and *M. flavescens* are negative.

b. MEDIUM

Lowenstein-Jensen slants.

c. REAGENT

20% ferric ammonium citrate, aqueous. Dispense in small containers. Autoclave. If solution becomes cloudy, discard and prepare fresh.

d. CONTROLS

Uninoculated medium (negative).
Medium inoculated with *M. fortuitum* (positive).

e. PROCEDURE

(1) Inoculate the medium with one drop of a barely turbid aqueous suspension of organisms.

(2) Incubate until obvious growth appears. Incubate control medium with test cultures.

(3) To the culture growth and to the uninoculated control medium, add the sterile citrate solution. Use one drop for each 1 ml of Lowenstein medium in the slant.

(4) Reincubate the cultures for a maximum of 21 days. Read weekly.

f. **RESULT**

A positive reaction is seen as the appearance of a rusty brown color in the colonies and a tan discoloration of the medium.

Reference:

Wayne, L.G. and Doubek, J.R. 1968. Diagnostic key to mycobacteria encountered in clinical laboratories. Appl. Microbiol. 16(6): 925-931.

4. MacConkey Agar

a. **CULTURES**

Inoculate all rapid growers onto MacConkey agar. *M. fortuitum* and *M. chelonei* show growth and sometimes a color change in 5-11 days.

b. **EQUIPMENT**

3 mm loop.
Petri dish turntable.

c. **MEDIUM**

MacConkey agar without crystal violet, 20 ml per plate.

d. **CONTROLS**

An uninoculated plate of medium (negative).
A plate inoculated with *M. phlei* (negative).
A plate inoculated with *M. fortuitum* (positive).

e. **PROCEDURE**

(1) Label plates to correspond with the culture numbers.

(2) Place the plate medium-side down on the turntable. Place a 3mm loopful of a 7-day liquid culture in the center of the plate touching the surface of the

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medium. Spin the turntable and move the loop slowly along the surface of the medium toward the periphery of the plate. This produces inoculum in the form of a spiral.

(When a turntable is not available, plates may be streaked as for isolated colonies, but the results by this method are not as conclusive.)

(3) Incubate plates in a Petri dish cannister, with the top loose, at 35°-37° C, WITHOUT ADDITIONAL CO_2 IN THE ATMOSPHERE.

(4) Read at 5 days and 11 days.

f. RESULTS

Positive = growth; a change in color may also occur.
Negative = no growth.

M. fortuitum and *M. chelonei* usually grow along the entire length of the spiral. With heavy inoculum other rapid growers may show some growth near the beginning of the spiral.

References:

Jones, W.D. Jr. and Kubica, G.P. 1964. The use of MacConkey's agar for the differential typing of *Mycobacterium fortuitum*. Amer. J. Med. Tech. 30: 187-190.

Kubica, G.P. and Vitvitsky, J. 1974. Comparison of two commercial formulations of the MacConkey agar test for mycobacteria. Appl. Microbiol. 27: 917-919.

5. Niacin

a. CULTURES

Test all nonchromogenic mycobacteria for niacin production. (From 7H-10 medium,* only the rough nonchromogenic strains need to be tested for niacin.) A culture must have at least 50-100 colonies with growth 3 to 4 weeks old. *M. tuberculosis* and the more rare *M. simiae* are usually niacin positive. Most other mycobacteria are niacin negative.

b. EQUIPMENT

Sterile screw-cap test tubes, 16 x 125 mm. Label with numbers to correspond with cultures being tested.
Sterile pipettes, 1 ml and 5 ml.

*The niacin test from cultures on 7H-10 medium is satisfactory when the 7H-10 contains 0.1% aspartic acid (potassium salt).

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c. REAGENTS*

Sterile distilled water or 0.85% saline.

4% aniline. Preparation:

To 96 ml of 95% ethyl alcohol, add 4.0 ml colorless aniline. Store in a brown bottle in the refrigerator. If the solution turns yellow, discard and prepare fresh solution.

10% cyanogen bromide. Preparation:

Dissolve 5 g cyanogen bromide in 50.0 ml distilled water. Store in a tightly capped brown bottle in the refrigerator. If a precipitate forms upon cooling, warm to room temperature to dissolve. Prepare in small quantities because the reagent is volatile and loses strength. Weak solutions may give false negative readings.

CAUTION: *Cyanogen bromide is tear gas and should be used in a well-ventilated safety cabinet. Cyanogen bromide also may hydrolyze in acid solution to hydrocyanic acid; discard niacin tests into a germicidal solution made alkaline by the addition of NaOH.*

d. CONTROLS

Reagent control on uninoculated medium (negative).
Reagent control on *M. avium (intracellulare)* culture (negative).
Reagent control on *M. tuberculosis* culture (positive).

e. PROCEDURE

(1) To the culture slant or quadrant on drug free medium, add 1.0 ml sterile distilled water or saline.

(2) If growth on the medium is completely confluent, puncture the surface so the liquid is in contact with the medium. Niacin, if present, is extracted from the medium, not from the bacterial growth.

(3) Allow the fluid to remain in contact with the culture medium for 15 minutes. When the culture is in a tube, place in a slanted position so the fluid layers over the colonies.

*A TB-niacin test strip is available commercially and may be used instead of the test reagents. Follow manufacturer's instructions.

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(4) Remove 0.5 ml of the liquid extract from the culture and place in a numbered, clean, screw-cap test tube.

(5) Add 0.5 ml of aniline (equal volume) to the extract. This should be colorless.

(6) Next, add an equal volume (0.5 ml) of cyanogen bromide.

(7) Observe for a yellow color which should appear immediately.

f. **RESULTS**

Positive = yellow color.
Negative = no color change.
For examples, see Figure 18.

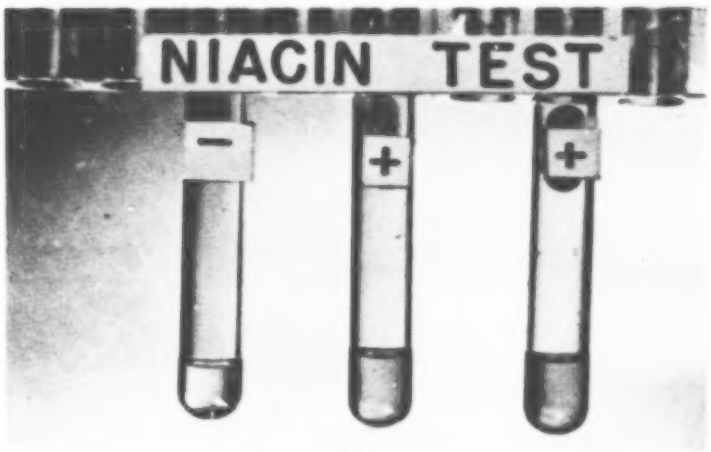

Figure 18. Niacin Test

References:

Kilburn, J.O., Stottmeier, K.D. and Kubica, G.P. 1968. Aspartic acid as a precursor for niacin synthesis by tubercle bacilli grown on 7H-10 agar medium. Amer. J. Clin. Path. 50(5): 582-586.

Konno, K. 1956. New chemical method to differentiate human-type tubercle bacilli from other mycobacteria. Science 124: 985.

Runyon, E.H., Selin, M.J. and Harris, H.W. 1959. Distinguishing mycobacteria by the niacin test. Amer. Rev. Tuberc. 79: 663-665.

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6. Nitrate Reduction

a. CULTURES

Test all mycobacterial cultures 3 to 4 weeks after visible colonies appear. Among the clinically significant mycobacteria, *M. tuberculosis*, *M. kansasii*, and *M. szulgai* are nitrate positive. Others that are positive include *M. flavescens*, most of the commonly saprophytic nonphotochromogens, and most rapidly growing species (notable exception is *M. chelonei*).

b. EQUIPMENT

Screw-cap test tubes (16 x 125 mm). Label with numbers to correspond to the cultures to be tested.
Sterile pipettes, capillary or 1 ml and 5 ml.
Inoculating spade or loop.
Water bath at 37°C or constant temperature block heater.

c. REAGENTS*

Substrate M/100 sodium nitrate in M/45 phosphate buffer, pH 7.0, prepared as follows:

$NaNO_3$	0.085 g
KH_2PO_4	0.117 g
$Na_2HPO_4 \cdot 12H_2O$	0.485 g
Distilled water	100.0 ml

Test Solutions:

No. 1 A 1:2 dilution of concentrated HCl (add 10 ml HCl to 10 ml H_2O).

NOTE: *Always add concentrated acid to water, never the reverse.*

No. 2 Dissolve 0.2 g sulfanilamide in 100 ml H_2O.

No. 3 Dissolve 0.1 g n-naphthylethylenediamine dihydrochloride in 100 ml distilled water.

Store reagents and substrate in the dark in the refrigerator. If precipitate forms or if reagents No. 2 and No. 3 change color, discard and prepare fresh solutions.

Distilled water, sterile.

Color standard for reading test. (Appendix C)

*The nitrite test strip available commercially detects the presence of nitrites. It gives satisfactory results only with strongly nitrate reducing cultures such as *M. tuberculosis*. Follow manufacturer's instructions.

d. **CONTROLS**

Reagent control without organisms (negative).
Reagent control with *M. tuberculosis* (positive).

e. **PROCEDURE**

(1) Place 3 or 4 drops of sterile distilled water in each sterile screw-cap test tube.

(2) Emulsify one loopful or spadeful of colonies from solid medium in the water.

(3) Add 2 ml of the $NaNO_3$ substrate solution to each test.

(4) Shake by hand to mix and incubate at 37°C for 2 hours. Remove from water bath.

(5) Add to each tube 1 drop of Reagent No. 1. Shake by hand to mix.

(6) Add to each tube 2 drops of Reagent No. 2.

(7) Add to each tube 2 drops of Reagent No. 3.

f. **RESULTS**

(1) If a red color develops, the test is positive. This color change may vary from pink to deep red.

Compare results with the color standard and record ± to 5+ according to the intensity of color change.

(2) If no color change occurs, confirm results. To confirm, add to all no-color-change tubes a small amount of powdered zinc. (Dip the tip end of a slightly moist applicator stick into dry zinc dust and shake the dust into the liquid.)

(a) Development of a red color following addition of zinc confirms that the no-color-change was a negative result.

(b) If no color change occurs upon adding zinc, the reaction was positive, with the nitrates reduced beyond nitrites into colorless components. In such cases, the test should be repeated to confirm the observation.

For examples, see Figure 19.

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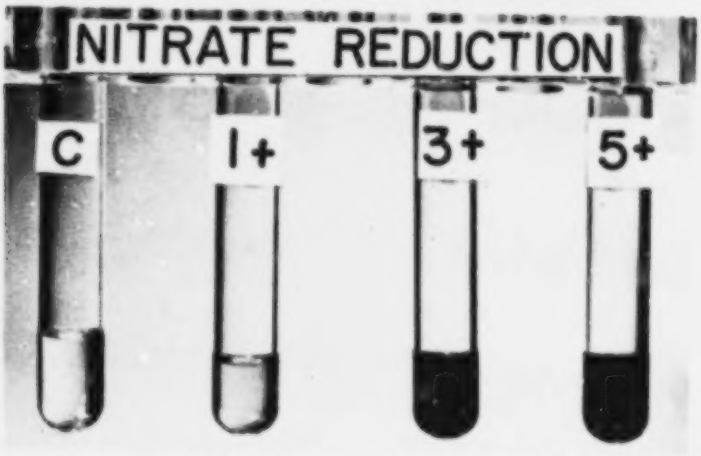

Figure 19. Nitrate Reduction Test (examples of weak and strong reactions).

Reference:

Virtanen, S. 1960. A study of nitrate reduction by mycobacteria. Acta. Tuberc. Scand. Suppl. 48: 1-119.

7. Pigment Production

a. CULTURES

Pigment production studies should be performed on:

(1) all slow growers that are nonchromogenic when incubated in the dark, and

(2) chromogenic cultures that have not been incubated continuously in the dark. The culture must be young, actively growing, and have isolated colonies. A change from nonchromogenic to chromogenic is characteristic of the photochromogens.

b. MEDIUM

Egg-base or agar-base.

c. INOCULUM

May be:
Diluted sediment of clinical specimen,
liquid culture (7-10 days) diluted, or
a dilute saline suspension of colonies taken from solid medium.

d. CONTROLS

The test procedure provides its own control.

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e. **PROCEDURE**

(1) Inoculate two tubes of egg-base or two plates of agar-base medium so that isolated colonies will be obtained.

(2) Shield one of the plates or tubes from light with black paper or aluminum foil.

(3) Incubate in the dark until visible colonies appear on the culture without the shield.

(4) Remove shield from the other culture. Record any pigmentation. If nonchromogenic, cover half the tube or plate and expose the other half of the culture to the light of a 30-60 watt lamp at a distance of 8-10 inches for 1 hour. Loosen the cap of the tube during exposure time.

(5) Replace shield. With cap still loose, reincubate for 6-12 hours or overnight.

(6) Compare pigmentation of colonies on exposed half of the culture to that on the unexposed portion.

f. **RESULT**

A change from nonchromogenic to lemon yellow indicates a photochromogen.

For examples, see Figure 10.

Reference:

Wayne, L.G. and Doubek, J.R. 1964. The role of air in the photochromogenic behaviour of *Mycobacterium kansasii*. Amer. J. Clin. Path. 42: 431-435.

8. **Pyrazinamidase Test**

In this test, *M. marinum* is positive in 4 days and *M. kansasii* is negative. *M. bovis* is negative at 4 and 7 days, whereas the *M. avium* complex and *M. tuberculosis* are positive in 4 days. This characteristic may be helpful in separating the weakly niacin positive *M. bovis* from *M. tuberculosis*, and in distinguishing *M. bovis* from members of the *M. avium* complex.

a. **MEDIUM**

Dubos broth base . . . . . 6.5 g
Distilled water . . . . . 1000.0 ml
Dissolve, add
Pyrazinamide . . . . . 0.1 g
Pyruvic acid, sodium salt . . . . . 2.0 g
Agar . . . . . 15.0 g

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Heat to melt agar. Dispense in 5-ml amounts to 16 x 125 mm screw-cap tubes. Autoclave 15 min/15 lb. Allow to harden upright, not slanted.

b. REAGENT

Ferrous ammonium sulfate, 1%. Prepare fresh, just before use.

c. CONTROLS

Uninoculated medium (negative).
Medium inoculated with *M. avium (intracellulare)* (positive).

d. PROCEDURE

(1) Place a heavy inoculum from a fresh culture slant onto the surface of each of 2 tubes of medium. Use enough inoculum so that it may readily be seen with the naked eye.

(2) Incubate at 35°-37° C.

(3) After 4 days, remove one tube from the incubator and add 1 ml of freshly prepared ferrous ammonium sulfate solution to the tube.

(4) Place the tubes in the refrigerator to prevent growth of contaminants.

(5) After 4 hours, examine tubes for a pink band in the agar. To read, hold the tubes against a white background and use incident room light.

(6) After 7 days' incubation, remove the second tube from the incubator and develop as described in (3), (4) and (5).

NOTE: *If the 4-day test is positive, it is not necessary to hold the second tube for the 7-day test.*

d. RESULT

Positive = a pink band in the agar.
Negative = no pink band in the agar.

Reference:

Wayne, L.G. 1974. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. Amer. Rev. Resp. Dis. 109: 147-151.

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9. Rabbit Inoculation

Mycobacteria suspected of being *M. bovis* may usually be identified by their relative unresponsiveness in most *in vitro* tests. On occasion, an especially troublesome isolate may have to be inoculated into a rabbit(s); bovine tubercle bacilli generally produce progressive disease in this laboratory animal.

a. PROCEDURE

(1) Suspect cultures should be transferred from solid medium to Tween-albumin liquid medium.

(2) Incubate at 35°-37° C for 7 days, at which time growth should be turbid.

(3) Dilute liquid culture 1:10 with sterile distilled water or physiological saline.

(4) Inoculate 1 ml of the 1:10 dilution into the marginal ear vein of at least one 4- to 6-lb. rabbit.

(5) Plate the culture on 7H-10 or Dubos oleic acid-albumin agar to determine both viability of culture and numbers of living bacilli injected into the rabbit.

b. RESULTS

(1) Rabbits inoculated with *M. bovis* may die of generalized tuberculosis within 90 days. At autopsy, grossly visible lesions in lungs, spleen, and liver are indicative of *M. bovis*. Make impression smears of these organs and stain for acid-fast bacilli.

(2) *M. avium* may also prove fatal for rabbits, but generally the gross disease is neither as extensive nor as apparent as that caused by *M. bovis*. Smears of "apparently normal" spleens and livers from such rabbits may reveal many acid-fast bacilli, the characteristic Yersin reaction often associated with *M. avium*.

(3) Rabbits that survive 90 days should be sacrificed. Should autopsy reveal only a few isolated lesions in the lungs or kidneys, this may indicate *M. tuberculosis*.

(4) If the rabbit(s) die in less than 6 weeks, and the autopsy is negative for both suspected *M. bovis* and *M. avium*, inoculate other animals.

Reference:

Willis, H.S. and Cummings, M.M. 1952. Diagnostic and Experimental Methods in Tuberculosis. Chas. L. Thomas, Co., Springfield, Illinois.

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10. Sodium Chloride Tolerance

a. CULTURES

Transfer all positive cultures to 5% sodium chloride medium. This is a test to confirm rapid growers and the slow growing *M. triviale. M. flavescens* sometimes grows on sodium chloride.

b. MEDIUM

5% NaCl in ATS (American Trudeau Society) or Lowenstein-Jensen medium.
ATS or Lowenstein without NaCl as control.
(See page 135 for preparation of medium, or use commercially prepared medium.)

c. INOCULUM

0.1 ml of a barely turbid suspension of a liquid culture or growth from a slant suspended in sterile water or saline.

d. CONTROLS

The test procedure provides the control.

e. TEST

(1) Inoculate a slant of NaCl test medium and a control slant without NaCl with 0.1 ml inoculum.
(2) Incubate at 35°-37°C.
(3) Read once a week for growth.
(4) Cultures may be discarded after 4 weeks' incubation.

f. RESULTS

Positive = growth on the NaCl and growth on the control.
Negative = no growth on NaCl but growth on the control.

Reference:

Kestle, D.G., Abbott, V.D. and Kubica, G.P. 1967. Differential identfication of mycobacteria. II. Subgroups of Groups II and III (Runyon) with different clinical significance. Amer. Rev. Resp. Dis. 95: 1041-1052.

11. Tellurite Reduction

a. CULTURES

Inoculate all slow growing nonphotochromogenic cultures as soon as sufficient growth appears for transfer. *M. avium (intracellulare)* is positive at 3 days, whereas other nonphotochromogens commonly are negative at 3 days.

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b. MEDIUM

Prepare Middlebrook 7H-9 liquid with Tween 80. Do not use glycerol. Use only 5 ml of medium in 20 x 150 mm screw-cap tubes.

Label tubes according to culture numbers.

c. REAGENT

0.2% potassium tellurite.

To prepare:

(1) Dissolve 0.1 g potassium tellurite in 50 ml distilled water.

(2) Dispense in several tubes in 2 ml amounts.

(3) Autoclave 10 minutes.

(4) Store in the refrigerator.

d. CONTROLS

Uninoculated medium and reagent (negative).
Medium inoculated with an *M. avium* complex strain (positive).

e. PROCEDURE

(1) Inoculate the liquid medium with a spadeful of organisms from an actively growing culture.

(2) Incubate at 35°-37°C for *7 days.* Be certain growth is *heavily turbid.* Do not prolong incubation. If growth is not *heavily turbid,* and suspected strains of the *M. avium* complex are negative, repeat test with a fresh subculture having heavy growth.

(3) With a capillary pipette, add 2 drops of the potassium tellurite solution to each tube. Discard any unused tellurite solution rather than chance its contamination with extraneous microorganisms.

(4) Reincubate at 35°-37°C.

(5) On the third day following reincubation, read the test results. *Do not shake tubes.*

f. RESULTS

Positive = black metallic precipitate of tellurium.
Negative = no black precipitate.

For examples, see Figure 20.

Reference:

Kilburn, J.O., Silcox, V.A. and Kubica, G.P. 1969. Differential identification of mycobacteria. V. The tellurite reduction test. Amer. Rev. Resp. Dis. 99:94-100.

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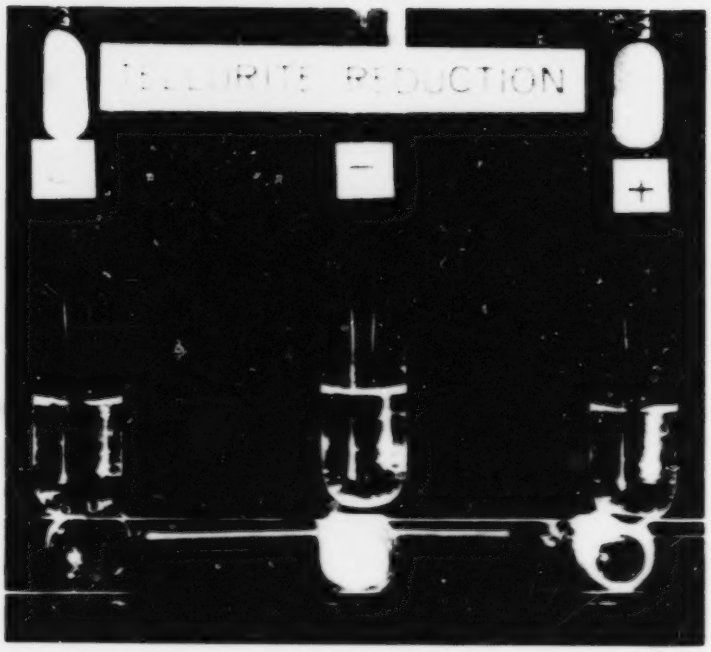

Figure 20. Tellurite Reduction Test

12. **Thiophen-2-Carboxylic Acid Hydrazide (TCH) Sensitivity**

This test is used to distinguish *M. bovis* from other non-chromogenic slow growing mycobacteria. *M. bovis* is sensitive to low concentrations of this compound. *M. tuberculosis* and other species of mycobacteria generally are resistant. Isoniazid resistant strains of *M. bovis* may also be resistant to TCH.

a. **MEDIUM***

Prepare two flasks of 7H-10 or Dubos Middlebrook agar with albumin complex. Use one as control medium, and to the other add filter sterilized TCH solution to make 10 mcg TCH/ml of medium. Dispense each into screw-cap test tubes or sectioned petri dishes.

This test may be run along with drug susceptibility tests using one quadrant of the plate for TCH.

b. **PROCEDURE:**

(1) Prepare a 7- to 10-day-old liquid culture as for rabbit inoculum. (OR prepare a barely turbid suspension of organisms from a drug-free medium.)

*An egg-base medium may be used. Add the required amount of TCH to the completed base, dispense, and inspissate.

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(2) Dilute with sterile saline or distilled water to 1:10 and 1:1000 (10^{-1} and 10^{-3}).

(3) With a sterile capillary pipette, inoculate one control and one TCH medium with three drops of the 10^{-3} dilution each; then inoculate a control and TCH medium each with three drops of the 10^{-1} dilution.

(4) Incubate 3 weeks in 5%-10% CO_2 atmosphere.

(5) Read in the same manner as drug susceptibility test plates, comparing growth on TCH with that on control medium.

c. RESULTS

Record the organism as sensitive if growth on the TCH medium is less than 1% of the growth on the control.

References:

Harrington, R. and Karlson, A.G. 1966. Differentiation between *M. tuberculosis* and *M. bovis* by in vitro procedures. Amer. J. Vet. Res. 27: 1193-1196.

Vestal, A.L. and Kubica, G.P. 1967. Differential identification of mycobacteria. III. Use of thiacetazone, thiophen-2-carboxylic acid hydrazide and triphenyltetrazolium chloride. Scand. J. Resp. Dis. 48: 142-148.

13. Tween 80 Hydrolysis (Degradation)

a. CULTURES

Inoculate all slow growers into Tween substrate as soon as sufficient growth appears for transfer.

This test separates the clinically significant scotochromogens and nonphotochromogens from the commonly saprophytic representatives of these groups. It also is an excellent confirmatory test for *M. kansasii*.

b. SUBSTRATE

To Prepare

(1) Combine:
100.0 ml M/15 phosphate buffer, pH 7.0.
0.5 ml Tween 80.
2.0 ml of a 0.1% aqueous neutral red stock solution, corrected for actual dye content.

(2) Dispense in 2.0 ml amounts into 16 x 125 mm screw-cap tubes.

(3) Autoclave 10 minutes at 121°C. This should be an amber colored liquid after autoclaving.

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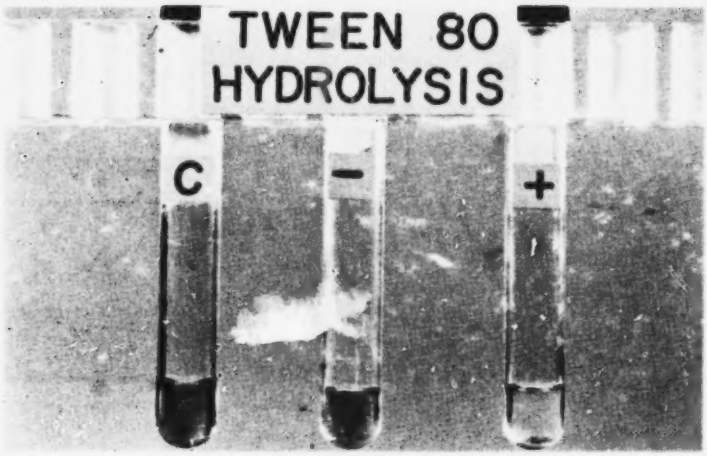

14. Urease

a. **CULTURES**

The scotochromogenic *M. scrofulaceum* is urease positive, whereas occasionally encountered pigmented strains of the *M. avium* complex, *M. xenopi*, and *M. gordonae* are negative.

b. **SUBSTRATE**

(1) Mix one part of Difco-Bacto urea agar base concentrate with 9 parts of sterile distilled water. Do not add agar.

(2) Dispense in 3-ml amounts into 13 x 100 mm sterile screw-cap tubes, or in 4-ml amounts in 16 x 125 mm screw-cap tubes.

c. **CONTROLS**

Uninoculated substrate (negative).

Substrate inoculated with *M. scrofulaceum*, *M. fortuitum*, or *M. gastri* (positive).

d. **PROCEDURE**

(1) Inoculate the liquid substrate with a spadeful of growth from an actively growing culture on solid medium.

(2) Incubate at 35°–37° C.

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(3) Observe for color change to pink or red. Discard after 3 days.

e. **RESULTS**

Positive = change to pink or red.
Negative = no color change.

References:

Toda, T., Hagihara, Y. and Takeya, K. 1960. A simple urease test for the classification of mycobacteria. Amer. Rev. Resp. Dis. 83: 757-761.

Wayne, L.G. 1974. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. Amer. Rev. Resp. Dis. 109: 147-151.

SECTION II G. PROBLEM CULTURES

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2. Contaminated Acid-Fast Growth 93
3. Mixed Acid-Fast Cultures 94

G. PROBLEM CULTURES

Adoption of new procedures frequently presents problems when a laboratory attempts to adapt them to the routine. These problems may stem from the omission of details in the published procedure and/or failure to follow directions precisely. One of the major problem areas appears to be contaminated cultures. Contamination may be so gross that no satisfactory result can be reported, or there may be acid-fast growth which is contaminated with other organisms.

The occurrence of two or more species of mycobacteria within a patient's specimen is not uncommon. Suspect such a mixed culture when any of the following occur:

More than one colony form is present.
Identification test pattern does not conform to that of any species.
Unusual drug susceptibility test results occur; for example, two or more colony forms appear on the control medium with one colony form on drug medium.

1. Gross Contamination

a. USE THE VORTEX MIXER PROPERLY. (SEE FIGURE 22.)

Improper use may create a safety hazard caused by aerosol and leakage and/or increased rate of culture contamination. A description of the proper use of the Vortex mixer follows:

Add digestant-decontaminant (NALC) to specimen in the centrifuge tube. Tighten cap. Hold the tube *above* the fluid level and *below* the cap with thumb and three fingers. For increased stability, the index finger may be placed on top of the cap.

Holding the tube at an angle, place it *gently* on the moving Vortex agitator-head and let swirl until liquefied.

When the tube is held in this manner, the fluid will swirl no higher than the level of grip by fingers and thumb and will remain below the level of the cap.

If the tube is incorrectly held, with thumb and fingers gripping near the cap, or if the tube is pressed too firmly on the Vortex mixer, fluid may swirl to the top and be retained on the inside of the cap. This retained fluid may not be decontaminated during the 15-minute decontami-

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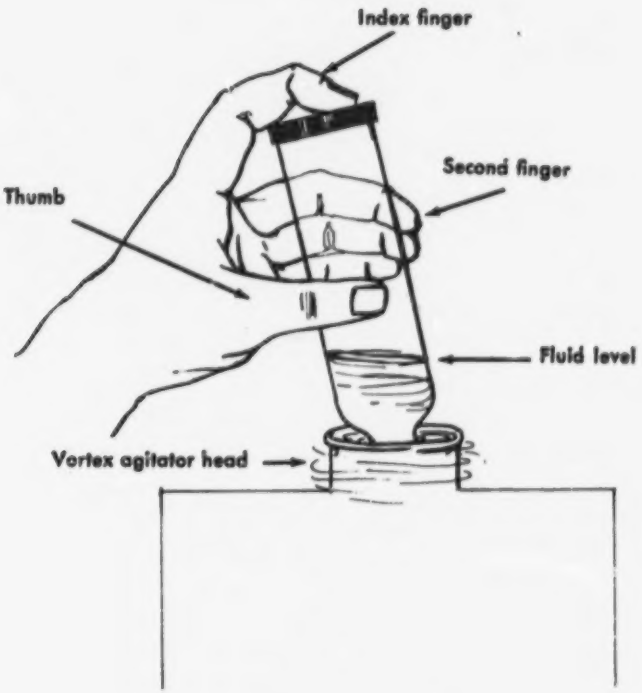

Figure 22. Proper Use of the Vortex Mixer

nation period. Then, too, during centrifugation some of this fluid in the cap may leak down the outside of the tube and produce an aerosol in the centrifuge.

b. IN DIGESTION, PERFORM THE DECONTAMINATION PROCEDURE PROPERLY.

(1) The NALC digestant should not be more than 24 hours old. Oxygen combines with NALC, making it an ineffective digestant.

(2) Check to see if Na citrate was added. Na citrate chelates heavy metal ions, which tend to bind the NALC and render it ineffective.

(3) If the specimen contains too much blood, the excess of iron of the hemoglobin binds the NALC and makes it impossible to digest the specimen.

(4) Mucoid materials should be completely digested. To aid mucolysis of "stubborn specimens," add a few extra crystals of N-acetyl-L-cysteine (10-30 mg) to the undigested specimen.

(5) More vigorous decontamination of digests may be needed. If so, increase the final concentration of NaOH in

digest from 2% to 3 or 4%. Do *not* increase decontamination time beyond 15 minutes. (Many mycobacteria are killed by over-decontamination.)

(6) Follow directions precisely.

c. CHECK STERILITY OF REAGENTS AND EQUIPMENT.

d. REDUCE AIR CONTAMINANTS IN THE ROOM TO A MINIMUM.

(1) Check air flow in biological safety cabinet.
(2) Place ultra filters over air intakes.
(3) Swab floor and work surfaces with disinfectant.
(4) Consider UV irradiation of upper air and counter surfaces when the room is not in use.

e. AVOID CONTAMINATION OF CULTURES DURING INCUBATION.

(1) Place plates with the medium side down in individual plastic bags. Seal with tape, rubber bands, or staples. (This prevents the spread of contaminants and makes the early reading of results more likely.)
(2) If it is the practice to incubate plates in bulk in large plastic bags, stack the plates no more than six high. Do not handle the bags excessively since opening and closing them to add or remove plates may introduce contaminants.
(3) Do not reuse plastic bags to incubate plates.
(4) Read plates first at 2 weeks. If earlier reading of a few cultures is necessary, use individual bags.
(5) Do not allow old cultures to remain in the incubator indefinitely.

2. **Contaminated Acid-Fast Cultures.**

Often it is possible to pick an isolated colony and streak it on to plates or tubes of medium, thereby obtaining a pure culture of acid-fast bacilli. When this is not possible, the following procedure may be used.

a. MATERIALS AND EQUIPMENT FOR DECONTAMINATION

Sterile screw-cap test tubes.
Sterile capillary pipettes.
Wire loop or straight needle.
Sterile distilled water.
4% NaOH, sterile.
2N HCl, sterile.
Phenol red or other indicator.
Microscope slides and stains.
Lowenstein and/or 7H-10 media.

b. PROCEDURE FOR DECONTAMINATION

(1) Number screw-cap tubes and slides for all cultures to be decontaminated.
(2) With a capillary pipette, place 4 drops of sterile distilled water in each tube.
(3) Pick a colony from the culture with a wire loop or spade. Suspend the organisms in the water by slowly grinding them against the side of the tube.
(4) Transfer a drop of this solution to a slide and stain for acid-fastness.
(5) Add 4 drops of 4% NaOH to the culture suspension in the tube.
(6) Tighten cap of tube and shake gently by hand to mix.
(7) Let stand at room temperature for 10 minutes. Shake occasionally. *Do not incubate at 37°C.*
(8) Add an indicator such as phenol red and neutralize with 2N HCl.
(9) Add 2 to 3 ml sterile distilled water.
(10) Inoculate one tube of Lowenstein medium with 0.1 ml of this suspension and one tube with a 1:10 dilution.
(11) With a wire loop, streak a plate of 7H-10 with a loopful of the undiluted suspension.
(12) When only one type of medium is available:
   a. Lowenstein: inoculate 2 additional tubes with the 10^{-2} and 10^{-4} dilutions.
   b. 7H-10: streak an additional plate with the 10^{-1} dilution.
(13) Incubate subcultures. Read at 24 hours, 1 week, and each week for 6 weeks.
(14) When growth appears, select isolated colonies for culture identification procedure. Make smears to determine acid-fastness and purity.

NOTE: *If the contaminant was not destroyed by NaOH, the decontamination process may be repeated by reversing the procedure. To do this, add the 2N HCl to the suspension of bacilli at step (5), and in step (8) neutralize with 4% NaOH.*

3. Mixed Acid-Fast Cultures

a. MATERIALS AND EQUIPMENT

Plates and tubes of medium
Loop or straight wire for picking colonies
Microscope slides and stains

b. PROCEDURE FOR SEPARATION:

(1) Label media and slides with culture numbers and the colony forms selected.

(2) Keep accurate records of all colony separations. Label media with culture numbers followed by letters (a, b, etc.) and keep colony descriptions in record book.

(3) Pick each colony form to separate media, and streak for isolated colonies. If the culture has confluent growth after incubation, it is difficult to determine separate colony forms.

(4) Make a smear and stain for acid-fastness.

(5) Incubate cultures and read at 24 hours, 1 week, and each week for 6 weeks.

(6) Examine growth for pure colony forms, and compare with records of original colony descriptions.

(7) One colony form denotes a pure culture. However, cultures of mycobacteria other than *M. tuberculosis* and *M. bovis* frequently have more than one type of colony within a culture. Smooth colonies may be domed and spreading with an occasional rough variant. The number of rough variants in a culture usually increases on repeated culture transfer. Some smooth strains have been described as SmI, smooth interconvertible, since their colony forms are not stable and readily convert from one form to several forms upon transfer.

(8) An astute bacteriologist should be able to separate mixed strains when they occur.

SECTION III. DRUG SUSCEPTIBILITY TESTS

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A. INTRODUCTION

Drug susceptibility testing is one of the most difficult procedures to standardize for the diagnostic mycobacteriology laboratory.

Briefly, reasons for these difficulties are:

1. Variation in stability of drugs under different methods of sterilization and storage. (See Table VII.)
2. Alteration in the antimycobacterial activity of various drugs when incorporated into different media. (See Table VIII.)

The procedure now recommended by the CDC and in general use by the Veteran's Administration and Armed Forces is an economical version of the proportion method of Canetti et al. In the proportion method, the laboratory must determine that proportion of the bacterial population which is resistant to the concentration of drugs used for testing. When 1% or more of the organisms which grow on the control are observed to grow on a particular drug, then, for clinical purposes, that particular drug is not, or soon may not be, valuable for the treatment of a given patient.

Table VII

Percent of Active Drug Remaining after Autoclaving, Filtering, and Freezing.

Drug at 10,000mcg./ml.	Method of sterilization				Stored at −20°C.		
	Autoclave	Seitz	Glass	Millipore	3 mo.	5 mo.	6 mo.
Isoniazid	90	92	100	98	98	98	97
Streptomycin	90	88	95	95	95	92	90
PAS	100	90	100	100	85	80	75
Kanamycin	98	90	96	98	98	98	98
Ethionamide	90	80	90	90	95	88	85
Viomycin	94	95	98	95	98	95	94
Ethambutol	98	80	88	90	90	90	90
Cycloserine	0	98	98	98	85	75	65
Pyrazinamide	100	100	100	100	100	100	100
Rifampin	not tested			100	91	86	

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Table VIII

Range of Minimal Inhibitory Concentrations of Drugs, in 7H-10 and Lowenstein Medium, Tested with 20 Wild Strains of *M. tuberculosis*.

Drug	Medium	
	7H-10 mcg/ml	Lowenstein mcg/ml
Isoniazid	0.05- 1.5	0.05- 1.5
Streptomycin	1.0 - 2.0	1.0 - 5.0
PAS*	1.0 - 2.0	0.25- 0.5
Kanamycin	1.0 - 2.5	8.0 -12.0
Ethionamide	1.5 - 4.0	6.0 -15.0
Viomycin	2.0 - 5.0	15.0 -18.0
Ethambutol	2.0 - 6.0	2.0 - 6.0
Cycloserine*	20	20
Pyrazinamide	18-22	18-22
Rifampin	0.05- 0.2	2.5 - 5.0

*PAS and cycloserine seem to have lower minimal inhibitory concentrations in Lowenstein than in 7H-10 medium. Because of the bacteriostatic effect of these two drugs, minute colonies remain undetected on egg medium but they will be detected on 7H-10 medium.

The drug susceptibility test may be performed by either the direct or the indirect method.

The direct drug susceptibility test is performed on a specimen in which acid-fast bacilli can be demonstrated on a smear of the digested, concentrated specimen. The inoculum for the test is the specimen concentrate diluted according to the number of acid-fast bacilli seen on the smear.

The indirect drug susceptibility test is performed by using a subculture from a primary culture as the inoculum. Although the direct test is preferred, the indirect test may be performed when one of the following situations occurs:

The smear was negative and the culture has growth.

The growth on the control of the direct test is inadequate for a reliable report.

A reference culture is sent in by another laboratory.

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For performing the tests, inoculate medium containing concentrations of the primary drugs and control medium with no drug. In the 7H-10 medium these are:

Isoniazid (INH) 0.2mcg/ml
Streptomycin (SM) 2.0mcg/ml
Para-aminosalicylic acid (PAS) . . . . . 2.0 mcg/ml
Control . . . . . no drug

When the mycobacteria show 1% or more resistance to these concentrations of drugs, inoculate media containing higher concentrations and/or other drugs. See Table IX.

Table IX
Concentration of drug in susceptibility test medium (7H-10)

Drug	mcg/ml
Isoniazid	0.2, 1.0, and 5.0
Streptomycin	2.0 and 10.0
PAS	2.0 and 10.0
Kanamycin	5.0
Ethionamide	5.0
Viomycin	5.0
Ethambutol	6.0
Cycloserine	20.0
Pyrazinamide (pH 5.5)*	50.0
Rifampin	1.0

*To adjust 7H-10 medium to the required acidity, pH 5.5, it is necessary to alter the ratio of the buffering phosphate salts. (Stottmeier, 1967.)

To determine the proportion of resistance, the number of colonies on the control and the number of colonies on the drug medium must be determined. If the growth is confluent (4+), it cannot be counted. Two dilutions of inocula, usually one-hundredfold apart, are planted onto duplicate plates of medium. This should give countable colonies on at least one of the controls. (See examples, Section D, Reporting Drug Susceptibility Tests.)

A test is considered valid when there are 50 or more colonies on at least one of the controls.

For preparation of media, see page 108.

B. DIRECT TEST

Materials and Equipment

Medium: 7H-10 in quadrant Petri plates.

2 plates per specimen, one for each dilution of inoculum.

Sterile saline, 4.5 ml volumes in screw-cap tubes.

Pipettes, capillary and 1 ml.

Procedure

1. Stain and read smears of concentrated clinical specimens, recording the average number of acid-fast bacilli found per oil immersion field. Example: After finding a field with acid-fast bacilli, read at least 20 additional fields and take an average. The bacilli in a sputum concentrate are often found in clumps.

2. Select dilutions of concentrate for inocula on the basis of positive Ziehl-Neelsen smear results as follows:

Microscopy	Inoculate
Less than 1 AFB/field	undilute and 10^{-2} (1:100)
1–10 AFB/field	10^{-1} and 10^{-3} (1:10 and 1:1000)
More than 10 AFB/field	10^{-2} and 10^{-4} (1:100 and 1:10,000)

3. Dilutions may be made by using 0.5 ml of the concentrate and 4.5 ml of sterile water or saline. Mix and transfer successively 0.5 ml to 4.5 ml for the required dilutions.

4. With a capillary pipette inoculate three drops onto each quadrant of the drug and control media. Inoculate one plate with the highest selected dilution and duplicate plate with the lower dilution.

   For patients who have been under treatment, it is advisable to inoculate one plate with undiluted inoculum. Organisms from such individuals may be stained but non-cultivatable due to the action of the drugs.

5. Place plates, medium side down, in individual polyethylene plastic bags and seal. Plates may be inverted for incubation after the inoculum has dried, or at the time of the first weekly reading.

6. Incubate in an atmosphere of 5%-10% carbon dioxide at 35°-37°C.

7. Plates in individual bags may be read weekly, but most strains

of *M. tuberculosis* will not produce visible colonies until the end of the second or third week.

8. Report at 3 weeks. However, some mycobacterial cultures may have mature growth and show definite drug resistance in less than 3 weeks; if so, they should be reported.

9. Cultures should not be discarded as negative until the end of the fifth or sixth week. Some few cultures, especially drug resistant strains, may require 4 to 5 weeks for microscopic colonies to appear even on the drug-free control. Because these are cultures from the specimen concentrate, such colonies may be the only growth obtained for identifying the culture.

10. On the drug-containing medium, any growth appearing after 3 weeks' incubation may not necessarily be comprised of resistant cells. Some drugs are no longer active in the medium after several days' incubation. If the drug is bacteriostatic rather than bacteriocidal, the mycobacteria may begin to multiply after the drug has become ineffective. As a rule, such colonies are small in comparison with those on the drug-free control.

11. Examine plates with the aid of a dissecting microscope using 30X-60X total magnification and transmitted light. Sometimes colonies are visible macroscopically on the control but are only visible microscopically on the drug-containing medium. Do not discard a plate as negative without reading it with the aid of magnification.

12. Record bacterial growth on the control and drug medium as follows:

Confluent (500 or more colonies)	++++ (4+)
Almost confluent (200-500 colonies)	+++ (3+)
100-200 colonies	++ (2+)
50-100 colonies	+ (1+)
Less than 50	Actual Count

13. Record colony characteristics on the control medium.

14. Report results obtained with both dilutions and calculate percent resistance. (See D. Reporting the Drug Susceptibility Test.)

C. INDIRECT TEST

Whenever possible, the direct drug susceptibility test procedure should be used. Under circumstances requiring the indirect drug susceptiblity test, growth from a culture of mycobacteria on

drug-free liquid or solid medium is used as inoculum. For the test to be valid the inoculum must be large enough for good growth (50-100 colonies on the control quadrant) but not large enough to simulate drug resistance due to the growth of spontaneously occurring drug resistant mutants.

TO PREPARE INOCULUM FROM GROWTH ON SOLID MEDIUM:

1. Remove approximately 2-5 mg of growth from drug-free medium. Pick a small portion of each colony, if possible.
2. Transfer to a sterile 16 x 125 mm screw-cap tube containing 6-8 glass or plastic beads* and 3 ml of Tween-albumin medium.
3. Homogenize the cells on a test tube mixer for 5-10 minutes.
4. Allow the large particles to settle. Withdraw the supernate and adjust the density to approximately MacFarland No. 1 with sterile distilled water or 0.85% saline.**
5. Dilute to 10^{-2} and 10^{-4} in sterile distilled water or 0.85% saline.
6. Proceed as for direct test for inoculation, incubation, reading, and reporting. Be sure to indicate "indirect test" in the report.

TO PREPARE INOCULUM WITH TWEEN-ALBUMIN LIQUID CULTURE

1. Transfer a part of each colony to Tween-albumin medium, such as Dubos or 7H-9.
2. Incubate for 7 days or until as turbid as the MacFarland No. 1 standard.
3. Dilute to 10^{-3} and 10^{-5} in sterile water or saline.
4. Inoculate the two dilutions, incubate, read, and report as described for the direct test.

D. REPORTING

1. The report should contain:
   The type of test, DIRECT or INDIRECT.
   The amount of growth on the control medium.
   The amount of growth on the drug medium.
   The concentration of drug in the medium.
   A rough calculation of the percent of the bacilli resistant to the drug. Do not report RESISTANT or SENSITIVE.

*Glass beads 1-2 mm in diameter or high heat polystyrene plastic beads such as Styron (R) 700, 27 clear, No. 7, from Dow Chemical Co.

**Prepare MacFarland No. 1 Standard by adding 0.1 ml of 1% $BaCl_2$ to 9.9 ml of 1% H_2SO_4.

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2. This formula may be used to calculate percent:

$$\frac{\text{Number of colonies on the drug}}{\text{Number of colonies on the control}} \times 100 = \%\ \text{resistance}$$

Example:

Test a.

	Growth at undilute	Growth at 10^{-2}	% Resistant
Control	4+	150	
INH 0.2 mcg	1+	9	6%
SM 2.0 mcg	0	0	0%
PAS 2.0 mcg	0	0	0%

$$\frac{9 \text{ colonies}}{150 \text{ colonies}} \times 100 = 6\%$$

In this test 6% of the organisms are resistant to 0.2 mcg/ml INH.

Test b.

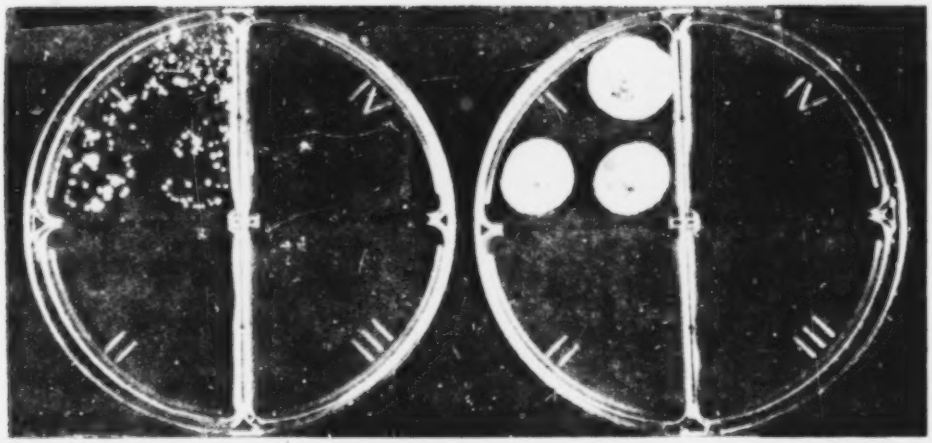
	Growth at 10^{-1}	Growth at 10^{-3}	% Resistant
Control	4+	60	
INH 0.2 mcg	120c	0	2%
SM 2.0 mcg	0	0	0%
PAS 2.0 mcg	0	0	0%

The 10^{-1} inoculum in test b. gave 4+, or colonies too numerous to count. The hundredfold dilution gave 60 countable colonies. Therefore 4+ = 60 x 100, or 6000 colonies. With this figure the proportion of resistant organisms may be calculated as 2% resistant to 0.2 mcg/ml INH.

$$\frac{120}{6000} \times 100 = 2\%$$

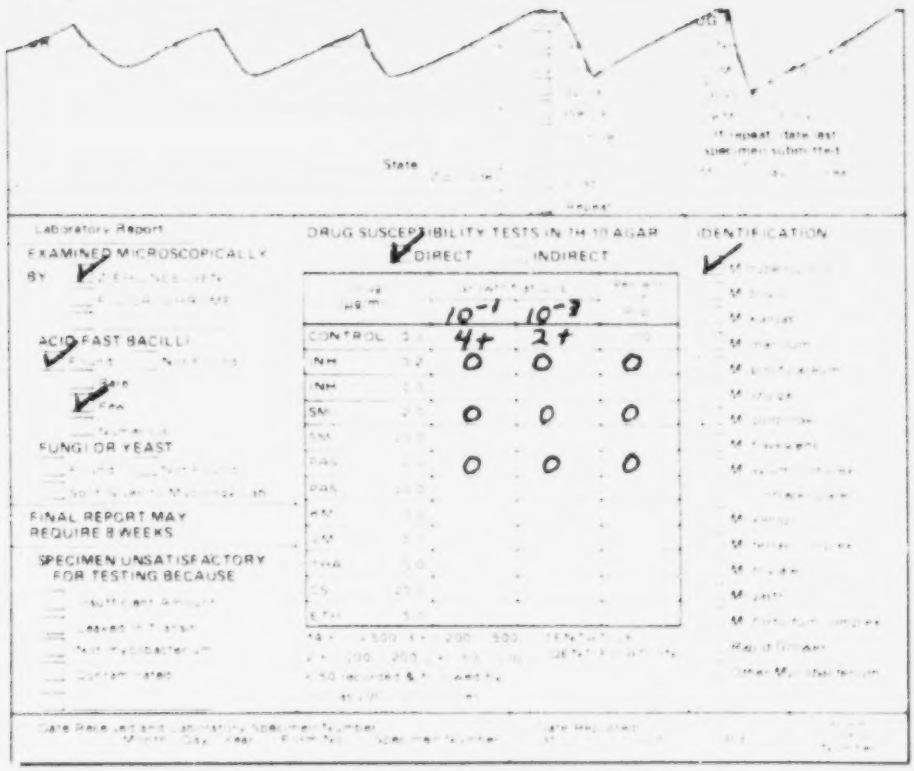
For other examples, see Figures 23-26.

CON. PAS 2.0



INH 0.2 SM 2.0

The organisms inoculated onto these plates were susceptible to 0.2 mcg. ml. INH (isoniazid) 2.0 mcg. ml. SM (streptomycin) and 2.0 mcg. ml. PAS (para-amino salicylic acid).



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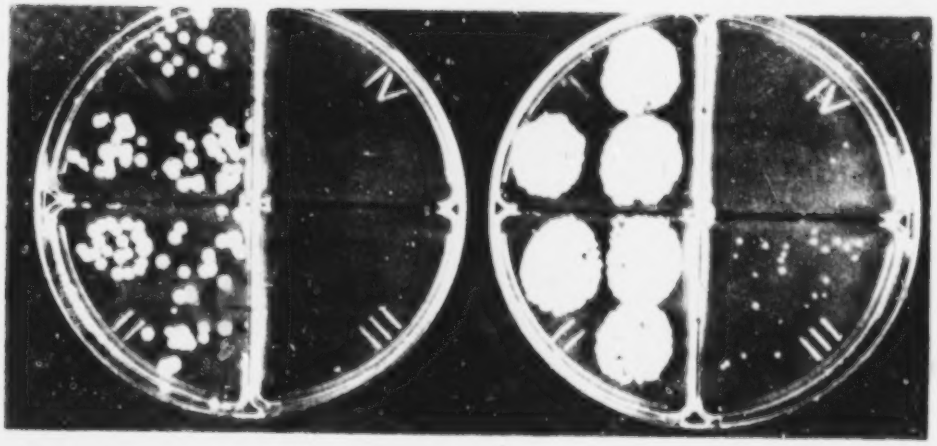
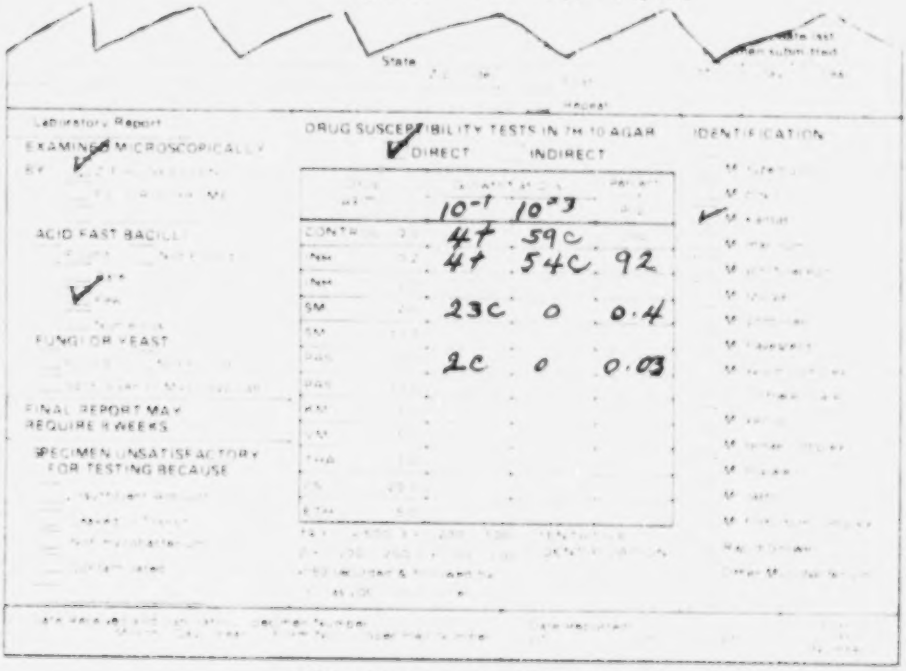
CON. PAS 2.0



INH 0.2 SM 2.0

The inoculum 10⁻¹ on the right shows 47 growth on the control and INH quadrants, 23 [illegible] on SM and 2 on PAS. A 100-fold dilution 10⁻³ of the inoculum on a second drug plate [illegible] 54 on the control, 54 on INH and none on SM and PAS. Hence 54 59 gives 92 [illegible] INH. The [illegible] resistant to SM and PAS multiply 59 × 100 = 5900 colony [illegible] quadrant of the 10⁻¹ plate.

23 5900 × 100 = 0.4% R to SM
2 5900 × 100 = 0.03% R to PAS



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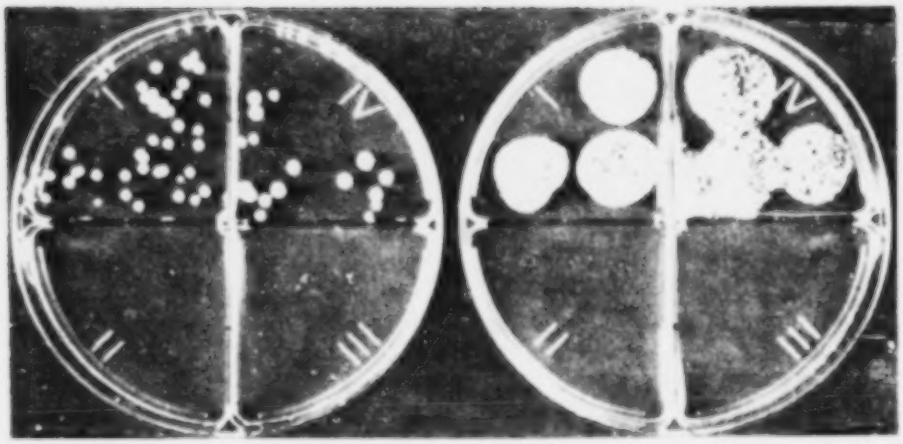
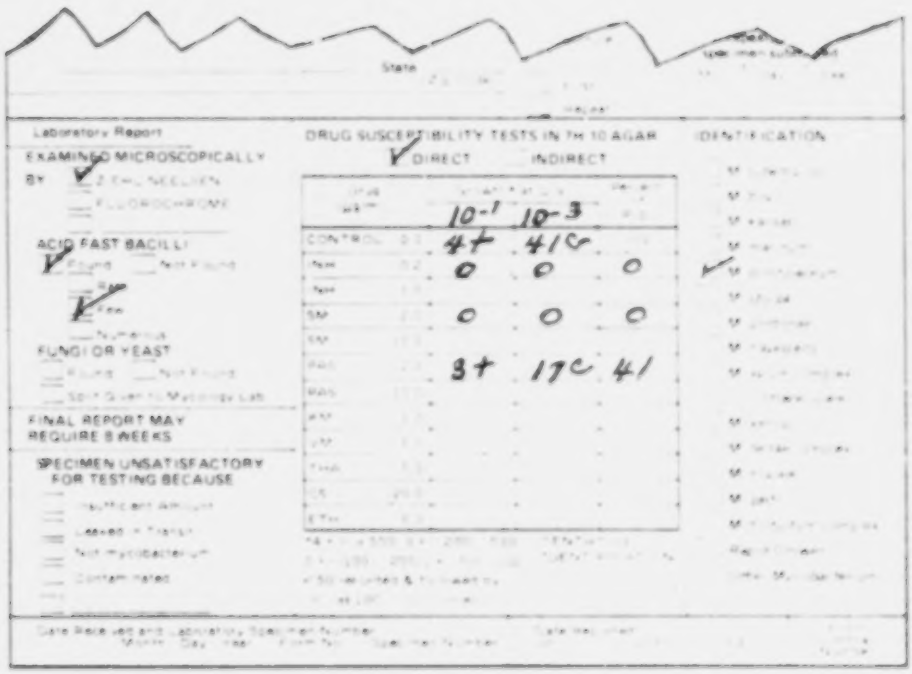
CON. PAS 2.0



INH 0.2 SM 2.0

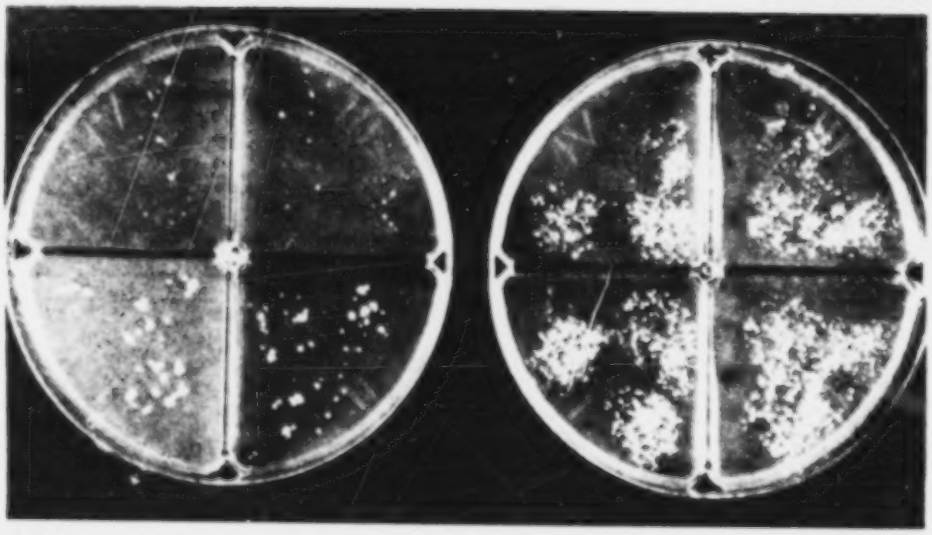
The inoculum on the plate at the right (10⁻¹) is too heavy to determine actual colony counts. In the hundredfold dilution of inoculum (10⁻³) on the plate to the left we count 41 colonies on the control and 17 on the PAS quadrant. Hence, the apparent resistance to PAS observed in the heavily inoculated plate is a true one, and approximately 41% of the population was resistant to PAS. 17 : 41 :: 100 : 41%.



SAMPLE LABORATORY REPORT FORM

PAS 2.0

INH 0.2



CON.

SM 2.0

There are innumerable colonies on all quadrants, even the more heavily inoculated plate. To compute more exactly the percentage resistance, there are 48 colonies on the control, 32 on INH, 49 on SM, and 37 on PAS. The percentage resistance is 67 for INH, 100 for SM, and 77 for PAS.

37 48 - 100 77 R to PAS

32 48 - 100 67 to INH

49 48 - 100 100 R to SM

State

Laboratory Report

EXAMINED MICROSCOPICALLY BY

ACID FAST BACILLI

FUNGI OR YEAST

FINAL REPORT MAY REQUIRE 8 WEEKS

SPECIMEN UNSATISFACTORY FOR TESTING BECAUSE

DRUG SUSCEPTIBILITY TESTS IN 7H 10 AGAR

DIRECT INDIRECT

IDENTIFICATION

	10°	10-2	
CONTROL	3+	48c	
INH	2+	32c	67
SM	3+	49c	100
	2+	37c	77

SAMPLE LABORATORY REPORT FORM

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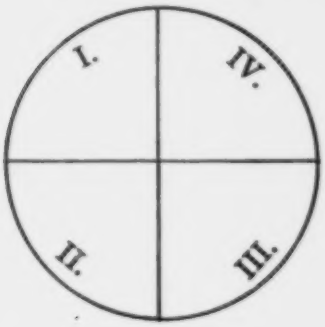
E. PREPARATION OF MEDIUM



Primary Drug Plates

Quadrant I.	Control
Quadrant II.	INH 0.2 mcg/ml
Quadrant III.	SM 2.0 mcg/ml
Quadrant IV.	PAS 2.0 mcg/ml

1. To prepare one set of quadrant plates using known concentrations of stock drug (see Table X):

   Prepare four flasks of 7H-10 medium base with 180 ml each. Cool to 50°–52°C.

   Four tubes of OADC, 20 ml each, warmed to room temperature.

   Sterile solutions of drug (See G. Preparation of Drugs for Susceptibility Test Media).

Table X
Preparation of Primary Drug Medium

Drug Medium Concentration		To Volume Of Medium	Add	Of Stock Drug
Control		200 ml	0	0
INH	0.2 mcg/ml	200 ml	0.4 ml	100 mcg/ml
SM	2.0 mcg/ml	200 ml	0.4 ml	1000 mcg/ml
PAS	2.0 mcg/ml	200 ml	0.4 ml	1000 mcg/ml

   a. To flask No. 1 add 20 ml sterile OADC enrichment. Mix well. Immediately dispense 5-6 ml of medium into quadrant I of each plate. (Control)

   b. To flask No. 2 add 20 ml sterile OADC and 0.4 ml of sterile 100 mcg/ml solution of INH. Mix and immediately dispense 5-6 ml into quadrant II of each plate. (0.2 mcg/ml)

   c. To flask No. 3 add 20 ml sterile OADC and 0.4 ml of sterile 1000 mcg/ml solution of SM. Mix well and immediately dispense 5-6 ml into quadrant III of each plate. (2.0 mcg/ml)

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d. To flask No. 4 add 20 ml sterile OADC and 0.4 ml of sterile 1000 mcg/ml solution of PAS. Mix well and dispense 5-6 ml into quadrant IV of each plate. (2.0 mcg/ml)

For higher concentration of primary drugs and concentration of other antituberculosis drugs, see Table XI.

Table XI
Preparation of Other Antituberculosis Drug Media

Drug Medium* Concentration		To Volume Media	Add	Of Stock Drug
Isoniazid	1.0 mcg/ml	200 ml	0.2 ml	1000 mcg/ml
Isoniazid	5.0 mcg/ml	200 ml	1.0 ml	1000 mcg/ml
Streptomycin	10.0 mcg/ml	200 ml	2.0 ml	1000 mcg/ml
PAS	10.0 mcg/ml	200 ml	2.0 ml	1000 mcg/ml
Kanamycin	5.0 mcg/ml	200 ml	1.0 ml	1000 mcg/ml
Ethionamide	5.0 mcg/ml	200 ml	1.0 ml	1000 mcg/ml
Viomycin	5.0 mcg/ml	200 ml	1.0 ml	1000 mcg/ml
Ethambutol	6.0 mcg/ml	200 ml	1.2 ml	1000 mcg/ml
Cycloserine	20.0 mcg/ml	200 ml	0.4 ml	10,000 mcg/ml
Rifampin	1.0 mcg/ml	200 ml	0.2 ml	1000 mcg/ml

*Each plate must have a control quadrant without drug for accurate reading of test results.

2. A method of preparing susceptibility test medium by means of drug-impregnated paper discs was described by Wayne and Krasnow in 1966 and is also found in the second edition of the "Manual of Clinical Microbiology" (1974).

   Paper discs containing appropriate concentrations of the primary antituberculosis drugs are commercially available. The discs, containing standardized amounts of drug, are dispensed aseptically into individual quadrants of the petri dishes (see Table XII). Measured 5.0 ml amounts of drug-free 7H-10 or 7H-11 agar are then pipetted over the disc. Take care to center the disc in the quadrant. Allow the media to solidify. Incubate the plates overnight to permit uniform diffusion of drug and to confirm sterility of the medium. When 7H-11 medium is used, studies have shown that the PAS concentration in drug

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plates should be increased to 5 mcg or 10 mcg/ml to compensate for the amount of PAS inactivated by components of the 7H-11 medium.

Table XII
Distribution of Drug-Containing Discs for Susceptibility Tests

Plate No.	Quadrant No.	DRUG	mcg of Drug in Disc	Final mcg/ml Drug in Medium
1	I	(Control #1)	–	0
	II	Isoniazid	1	0.2
	III	Isoniazid	5	1.0
	IV	Ethambutol	25	5.0
2	I	(Control #2)	–	0
	II	Streptomycin	10	2.0
	III	Streptomycin	50	10.0
	IV	Rifampicin	5	1.0
3	I	p–Aminosalicylic acid	10	2.0
	II	p–Aminosalicylic acid	50	10.0
	III		–	–
	IV		–	–

F. STORAGE OF DRUG MEDIUM

All media should be stored in the dark in sterile containers. Medium containing the following drugs may be stored at room temperature for 2 weeks without appreciable loss of drug potency:

Isoniazid
Streptomycin
PAS
Kanamycin
Viomycin
Ethionamide
Ethambutol
Rifampin

In the refrigerator these drugs remain stable in 7H-10 medium for as long as 2 months, but because stored media dehydrates, drug plates should be used within 1 month of preparation. Other problems such as lack of refrigerator space and contamination may be encountered, making it impractical to prepare a 2-month supply.

G. PREPARATION OF DRUGS FOR MEDIUM

1. Drug Potencies:

The true potency of the drug is the number of micrograms of active drug per milligram total weight of the product. Not all antimicrobial drugs have been isolated in pure form, and a portion of their weight may be due to impurities or to the sulfate or other radical component of the molecule. The potency should be stated on the vial, otherwise it is necessary to consult the manufacturer for this information. Each lot of partially purified drugs will vary from previous ones, and the potency of one lot may not be the same as that of another lot.

2. Stock solutions

Prepare stock solution and media the same day.

a. Drugs may be obtained in the sterile lyophilized form. Instructions accompanying the vial state the potency of the final solution when prepared according to directions. This sterile lyophilized drug reconstituted with sterile distilled water by aseptic technique requires no further sterilization. To obtain working stock solutions of the required concentration, dilute with sterile distilled water. Use a separate sterile pipette for each dilution. Solutions should be mixed well, and all measurements made accurately.

b. Bulk drug in powder form that must be weighed out from a stock vial and dissolved in distilled water requires sterilization. The membrane filter, such as Millipore, is recommended as a uniform method of sterilization. Some of the drugs lose potency when autoclaved or filtered through Seitz or sintered glass.

Millipore membrane filter models are available:

(a) Micro-syringe, Swinny or Swinnex (plastic) filter holders to attach to a 10-ml syringe having Luer fittings.

(b) Micro-analysis filter holder plus a 125-ml filtering flask.

The micro-syringe Swinny or Swinnex filters require no vacuum source and are easily cleaned by rinsing in tap water, soaking overnight in distilled water, and drying in the air.

The 25 mm HA filter, porosity 0.45 microns fits either of the micro models. Pre-filter type AP20 is placed on top of the filter paper.

1. When sterilizing, assemble the filter holder with pre-filter and filter paper in place.

2. Autoclave 10-15 minutes at 120°-121°C.
3. Allow autoclave pressure to rise slowly.
4. At the end of the sterilization period allow the autoclave to return slowly to normal. Sudden pressure changes may damage the delicate membrane of the filter.
5. Let filter cool to room temperature before use.

Filtering volumes less than 10 ml results in unnecessarily large loss of potency due to the drug being absorbed during the early part of the filtration process.

For calculating the weight of drug necessary to prepare 10 ml of a 10,000 mcg/ml solution, this formula may be used:

$$\frac{10{,}000}{\text{potency}} \times 10 = \text{milligrams to weigh}$$

Example: Potency of dihydro-streptomycin sulfate = 800 mg of active SM per 1000 mg

$$\frac{10{,}000}{800} \times 10 = 125$$

Dissolve 125 mg in 10 ml water for 10,000 mcg SM per ml.

Preparation of Isoniazid (INH) Solution

(10 mg isoniazid = 10 mg INH)

1. Dissolve 100 mg (100,000 mcg) isoniazid in 10 ml distilled water. This is a 10,000 mcg/ml solution.
2. Sterilize by Millipore filter.
3. Add 1 ml to 9 ml sterile distilled water. This is 1000 mcg/ml solution.
4. Add 1 ml of 1000 mcg/ml to 9 ml sterile water. This is 100 mcg/ml solution.
5. Add 0.4 ml of 100 mcg/ml to 200 ml medium to obtain a final concentration of 0.2 mcg INH per ml of medium.

Preparation of Streptomycin (SM) Solution

(12.5 mg dihydro-streptomycin sulfate = 10 mg SM.)

1. Dissolve 125 mg of dihydro-streptomycin sulfate in 10 ml distilled water. This is a 10,000 mcg/ml solution.
2. Sterilize by Millipore filter.

3. Add 1 ml to 9 ml sterile distilled water. This is 1000 mcg/ml solution.
4. Add 0.4 ml of 1000 mcg/ml solution to 200 ml of medium to obtain a final concentration of 2.0 mcg SM per ml of medium.

Preparation of PAS Solution

(5.7 g sodium p-aminosalicylate = 5 g PAS)

1. Dissolve 114 mg sodium p-aminosalicylate in 10 ml distilled water. This is a 10,000 mcg/ml solution.
2. Sterilize by Millipore filter.
3. Add 1 ml to 9 ml sterile distilled water to obtain 1000 mcg/ml solution.
4. Add 0.4 ml of 1000 mcg/ml to 200 ml medium to obtain a final concentration of 2.0 mcg PAS per ml medium.

Preparation of Kanamycin (KM) Solution

(1000 mg kanamycin sulfate = 832 mg KM)

1. Dissolve 121.5 mg kanamycin sulfate in 10 ml distilled water. This is a 10,000 mcg/ml solution.
2. Sterilize by Millipore filter.
3. Add 1 ml to 9 ml sterile distilled water. This is 1000 mcg/ml solution.
4. Add 1.0 ml of 1000 mcg/ml to 200 ml medium to obtain a final concentration of 5 mcg of KM per ml medium.

Preparation of Ethionamide (THA) Solution

(610 mg ethionamide hydrochloride = 500 mg THA)

1. Dissolve 122 mg of ethionamide hydrochloride in 10 ml distilled water. This is a 10,000 mcg/ml solution.
2. Sterilize by Millipore filter.
3. Add 1 ml to 9 ml sterile distilled water. This is a 1000 mcg/ml solution.
4. Add 1 ml of 1000 mcg/ml to 200 ml medium to obtain a final concentration of 5 mcg THA per ml medium.

Prepration of Viomycin (VM) Solution

(12.2 mg viomycin sulfate = 10 mg VM)

1. Dissolve 122 mg of viomycin sulfate in 10 ml distilled water. This is a 10,000 mcg/ml solution.
2. Sterilize by Millipore filter.
3. Add 1 ml to 9 ml sterile distilled water. This is a 1000 mcg/ml solution.
4. Add 1 ml of 1000 mcg/ml to 200 ml of medium to obtain a final concentration of 5 mcg VM per ml of medium.

Preparation of Ethambutol (EMB) Solution

(10 mg d-ethambutol = 10 mg EMB)

1. Dissolve 100 mg d-ethambutol in 10 ml distilled water.
2. Sterilize by Millipore filter.
3. Add 1 ml to 9 ml sterile distilled water. This is a 1000 mcg/ml solution.
4. Add 1.2 ml of 1000 mcg/ml to 200 ml of medium to obtain a final concentration of 6 mcg EMB per ml of medium.

Preparation of Cycloserine (CS) Solution

(10 mg cycloserine = 10 mg CS)

1. Dissolve 100 mg cycloserine in 10 ml distilled water. This gives 10,000 mcg/ml.
2. Sterilize by Millipore filter.
3. Add 0.4 ml of 10,000 mcg/ml to 200 ml of medium to obtain a final concentration of 20 mcg CS per ml of medium.

Preparation of Rifampicin (Rifampin) Solution

(10 mg rifampin = 10 mg rifampin)

1. Dissolve rifampin according to manufacturer's instructions to make a 10,000 mcg/ml solution.
2. Sterilize by Millipore filter.
3. Add 1 ml to 9 ml sterile distilled water. This is a 1000 mcg/ml solution.
4. Add 0.2 ml of 1000 mcg/ml to 200 ml medium to obtain a final concentration of 1.0 mcg of rifampin per ml of medium.

For storage of drugs, see Table VII in the Introduction of this section.

H. LEVELS OF ANTITUBERCULOSIS AGENTS IN BODY FLUIDS

On occasion a patient whose organisms show *in vitro* susceptibility to drugs may fail to respond to treatment with these agents. There may be several explanations for such "treatment failures"; however, these will not be discussed here. Insufficient serum levels of the drug(s) may be one explanation for this phenomenon, and this is mentioned only because the bacteriologist may be asked to determine the level of certain antituberculosis agents in various body fluids. If the clinician should request serum level determinations of antituberculosis drugs, relatively simple biological and chemical methods of drug assay are described by Stottmeier et al.

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SECTION IV. RECORDS AND REPORTS

The laboratory worker is responsible for keeping accurate records and reporting the actual results obtained by laboratory procedures. The worker does not interpret the results, since the physician has other information about the patient and is responsible for making the diagnosis. With careful observation and the test results, the laboratory worker should be able to make some distinction between the clinically significant and the clinically insignificant mycobacteria.

For laboratory results to be useful to the clinician in the diagnosis and treatment of the patient, uniform methods of reporting laboratory procedures are essential. Since laboratory tests in mycobacteriology are time-consuming, interim reports should be sent. A recommended reporting schedule follows:

First, report smear results as soon as they are available. This report should include: (1) the staining method used and (2) some indication of the number of acid-fast bacilli seen on the smear.

Later, at 3 to 4 weeks, report: (1) results of direct drug susceptibility tests, (2) amount of growth on the culture, and (3) a tentative or final identification of the species isolated.

Send final report upon identification of the species. Cultures should be held 6 to 8 weeks before they are reported as negative.

For examples of reports, see Figures 23-28.

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Figure 27
SAMPLE LABORATORY REPORT FORM

LABORATORY (Name)

Please print with BLACK MEDIUM SOFT lead pencil only

FORM (Name)

Form No.

Patient's Name: John Doe | Age: 69 | Sex: M | Race: W

Patient's Address: Somewhere U.S.A | County Code

Patient's Social Security Number or Occupation

Physician Code

This is your return address

DR. J. J. JAY

TB Sanatorium

Somewhere Stated KA (Zip Code)

To be filled in by physician

Date Specimen Collected: Mo. | Day | Year

SPECIMEN: Sputum:
- [ ] Natural
- [x] Nebulized
- [ ] Gastric
- [ ] Urine
- [ ] Spinal
- [ ] Pleural
- [ ] Culture
- [ ] ____
- [ ] First
- [ ] Repeat

IS PATIENT ON THERAPY? [x] Yes [ ] No

DRUG TESTS REQUESTED
- [x] INH [ ] VM [ ] ____
- [x] SM [ ] ETA [ ] ____
- [ ] PAS [ ] CS
- [ ] KM [ ] ETH [ ] ____

If repeat, date last specimen submitted: Mo. | Day | Year

Laboratory Report

EXAMINED MICROSCOPICALLY BY:
- [x] ZIEHL-NEELSEN
- [ ] FLUOROCHROME
- [ ] ____

ACID FAST BACILLI:
- [ ] Found [ ] Not Found
- [x] Rare
- [ ] Few
- [ ] Numerous

FUNGI OR YEAST:
- [ ] Found [ ] Not Found
- [ ] Split Given to Mycology Lab.

FINAL REPORT MAY REQUIRE 8 WEEKS

SPECIMEN UNSATISFACTORY FOR TESTING BECAUSE:
- [ ] Insufficient Amount
- [ ] Leaked in Transit
- [ ] Not mycobacterium
- [ ] Contaminated
- [ ] ____

DRUG SUSCEPTIBILITY TESTS IN 7H-10 AGAR
[x] DIRECT [ ] INDIRECT

Drug (µg/ml)		Growth* at Dils. 10^0	Growth* at Dils. 10^{-2}	Percent of Pop.
CONTROL	0.0	4+	50C	100
IHN	0.2	3+	30C	60%
INH	1.0	1+	2C	4%
SM	2.0	0	0	0
SM	10.0	0	0	0
PAS	2.0	0	0	0
PAS	10.0	0	0	0
KM	5.0			
VM	5.0			
THA	5.0			
CS	20.0			
ETH	5.0			

*4+ = > 500, 3+ = 200 – 500, 2+ = 100 – 200, 1+ = 50 – 100
<50 recorded & followed by "c", as 10C = 10 colonies

TENTATIVE IDENTIFICATION: ____

IDENTIFICATION
- [x] *M. tuberculosis*
- [ ] *M. bovis*
- [ ] *M. kansasii*
- [ ] *M. marinum*
- [ ] *M. scrofulaceum*
- [ ] *M. szulgai*
- [ ] *M. gordonae*
- [ ] *M. flavescens*
- [ ] *M. avium complex (intracellulare)*
- [ ] *M. xenopi*
- [ ] *M. terrae complex*
- [ ] *M. triviale*
- [ ] *M. gastri*
- [ ] *M. fortuitum complex*
- [ ] *Rapid Grower*
- [ ] *Other Mycobacterium*

Date Received and Laboratory Specimen Number: Month | Day | Year | Form No. | Specimen Number

Date Reported: 1st | 2nd | 3rd ✓

Form Control Number

Figure 28

SAMPLE LABORATORY REPORT FORM

LABORATORY (Name) | FORM (Name) | Form No.

Please print with BLACK MEDIUM SOFT lead pencil only

Patient's Name: MARY Doe | Age: 39 | Sex: F | Race: W

Patient's Address: BIG GAP U.S.A. | County Code

Patient's Social Security Number or Occupation

Physician Code

This is your return address

DR. ✓

State (Zip Code)

To be filled in by physician

Date Specimen Collected: Mo. | Day | Year

SPECIMEN: Sputum:
- ☐ Natural
- ☑ Nebulized
- ☐ Gastric
- ☐ Urine
- ☐ Spinal
- ☐ Pleural
- ☐ Culture
- ☐ ____
- ☐ First
- ☐ Repeat

IS PATIENT ON THERAPY? ☐ Yes ☐ No

DRUG TESTS REQUESTED
- ☑ INH ☐ VM ☐ ____
- ☑ SM ☐ ETA ☐ ____
- ☐ PAS ☐ CS
- ☐ KM ☐ ETH ☐ ____

If repeat, date last specimen submitted: Mo. | Day | Year

Laboratory Report

EXAMINED MICROSCOPICALLY BY:
- ☐ ZIEHL-NEELSEN
- ☑ FLUOROCHROME
- ☐ ____

ACID FAST BACILLI:
- ☐ Found ☐ Not Found
- ☐ Rare
- ☑ Few
- ☐ Numerous

FUNGI OR YEAST:
- ☐ Found ☐ Not Found
- ☐ Split Given to Mycology Lab.

FINAL REPORT MAY REQUIRE 8 WEEKS

SPECIMEN UNSATISFACTORY FOR TESTING BECAUSE:
- ☐ Insufficient Amount
- ☐ Leaked in Transit
- ☐ Not mycobacterium
- ☐ Contaminated
- ☐ ____

DRUG SUSCEPTIBILITY TESTS IN 7H-10 AGAR

☑ DIRECT ☐ INDIRECT

Drug (µg/ml)		Growth* at Dils. 10^{-1}	Growth* at Dils. 10^{-3}	Percent of Pop.
CONTROL	0.0	3+	1+	100
INH	0.2	0	0	0
INH	1.0			
SM	2.0	0	0	0
SM	10.0			
PAS	2.0	0	0	0
PAS	10.0			
KM	5.0			
VM	5.0			
THA	5.0			
CS	20.0			
ETH	5.0			

*4 + = > 500, 3 + = 200 – 500, 2 + = 100 – 200, 1 + = 50 – 100 <50 recorded & followed by "c", as 10C = 10 colonies

TENTATIVE IDENTIFICATION: ____

IDENTIFICATION
- ☑ *M. tuberculosis*
- ☐ *M. bovis*
- ☐ *M. kansasii*
- ☐ *M. marinum*
- ☐ *M. scrofulaceum*
- ☐ *M. szulgai*
- ☐ *M. gordonae*
- ☐ *M. flavescens*
- ☐ *M. avium complex (intracellulare)*
- ☐ *M. xenopi*
- ☐ *M. terrae complex*
- ☐ *M. triviale*
- ☐ *M. gastri*
- ☐ *M. fortuitum complex*
- ☐ *Rapid Grower*
- ☐ *Other Mycobacterium*

Date Received and Laboratory Specimen Number: Month | Day ✓ | Year | Form No. | Specimen Number

Date Reported: 1st | 2nd | 3rd ✓

Form Control Number

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APPENDIX A: EQUIPMENT

APPENDIX A:
EQUIPMENT

The items of equipment, glassware, chemicals, and other materials listed are generally found in most laboratories. Any specifications given are those required in the methods and procedures described in this manual.

1. GENERAL

Analytical balances
Autoclave, steam or electric
Beads, glass or plastic
Burner, Bunsen or Fisher
Carbon dioxide gauge, e.g., Fyrite or Burwell
Centrifuge with tachometer, 3000 rpm (1800-2400 *g*)
Desiccator, vacuum
Discard cans, splash proof
Discard pans, with covers
Filter pump
Filters, bacteriological (Seitz and Millipore)
Freezer: −20° to −40°C
Hand lens
Incubators, fitted for carbon dioxide
Inoculating spade or loop
Inspissator (for egg medium if used)
Interval timers
Microscope, dissecting, 10-50X for use with transmitted light
Microscope for fluorescence microscopy
Microscope with oil immersion lens
Mixer, test tube, high speed, e.g., Vortex or other
pH meter
Pipetting unit, Cornwall
Racks for specimen tubes
Refrigerator, 4° to 10°C
Safety equipment (See section 4. Safety, below)
Slide staining apparatus
Slide warming table, Ranson or equivalent
Temperature block heaters 37°C and 68°C
Thermometers
Turntable for inoculating plates
Water baths 37°C and 56°C

2. GLASSWARE

Bottles for stains
Cylinders, graduated, 25 ml to 1000 ml
Flasks, Erlenmeyer or other, 50 ml to 3000 ml
Flasks, Volumetric, 100 ml, 500 ml, 1000 ml
Pipettes

Capillary (Pasteur type)	5 ml graduated in 1/10 ml
1 ml graduated in 1/100 ml	10 ml graduated in 1/10 ml

Slides, microscope
27 x 75 mm frosted end, regular
27 x 75 mm for fluorescence microscopy
Tubes, screw-cap

13 x 100 mm	20 x 150 mm
16 x 125 mm	50 ml centrifuge, aerosol free

3. PLASTICS

Bags, Polyethylene
Centrifuge tubes, screw-cap, 50 ml, water-tight
Petri dishes
Plain 15 x 100 mm
I plate (2 sections) 15 x 100 mm
X plate (4 sections) 15 x 100 mm

4. SAFETY

Alcohol-sand flask (Figure 4)
Biological safety cabinet (hood)
Centrifuge safety cups, aerosol free
Discard pans with covers
Disinfectant for hands, 2% amphyl or other
Disinfectant, liquid phenol.
Hats, paper, disposable
Hospital gowns
Masks, disposable
Rubber gloves, disposable
Safety pipetting devices
Shoe covers, plastic, or autoclavable sneakers
Splash-proof containers for fluids (Figure 3)
Tissue grinder, Ten Broeck, hand
Ultrafilters for hood
Ultraviolet lamps in hoods
Ultraviolet single sheet paper sterilizer (or autoclave specimen slips in a paper bag)

APPENDIX B: CHEMICALS AND MEDIA BASES

APPENDIX B:
CHEMICALS AND MEDIA BASES

Only chemicals of the highest purity should be used. A few may be available from only one manufacturer.

CHEMICALS

Acridine Orange
Agar
Albumin, Bovine Fraction V
Alcohol, 95% ethyl
Ammonium hydroxide
Ammonium sulfate
Aniline
Asparagine
Auramine O
Barium chloride
Biotin
Brom thymol blue
Calcium chloride $\cdot 2H_2O$
Catalase, technical grade
Copper sulfate $\cdot 5H_2O$
Cyanogen bromide
Dextrose (glucose)
Disodium phosphate $\cdot 12H_2O$
Ferric ammonium citrate (green)
Ferrous ammonium sulfate
Fuchsin, basic, 94% dye content
Glycerol, reagent grade
Hydrochloric acid
Hydrogen peroxide (30% Superoxol)
Magnesium citrate
Magnesium sulfate $\cdot 7H_2O$
Malachite green oxalate
Methylene blue chloride
Monopotassium phosphate (anhydrous)
Monosodium glutamate
N-acetyl-L-cysteine powder
Neutral red
N-naphthylethylenediamine dihydrochloride

Oleic acid
Phenol crystals, reagent grade
Phenol liquid
Phenolphthalein
Phenolphthalein disulfate tripotassium
Phenolsulfonphthalein, sodium salt (phenol red)
Potassium permanganate
Potassium tellurite
Potato flour or potato starch
Pyrazinamide
Pyridoxine hydrochloride
Pyruvic acid, sodium salt
Sodium carbonate
Sodium chloride
Sodium citrate $\cdot 2H_2O$
Sodium hydroxide
Sodium nitrate
Sulfuric acid
2-thiophene carboxylic acid hydrazide (TCH)
Trisodium phosphate
Tween 80®
Zephiran (17% benzalkonium chloride)
Zinc metal powder, (dust)
Zinc sulfate $\cdot 7H_2O$

MEDIA BASES

Dubos broth base with ADC enrichment
MacConkey agar (no crystal violet)
Middlebrook 7H-9 broth base with ADC enrichment
Middlebrook 7H-10 agar base with OADC enrichment
Urease agar base, Difco-Bacto
Wayne Sulfatase agar

APPENDIX C: COLOR STANDARDS

APPENDIX C:

COLOR STANDARDS

Arylsulfatase Test Result Standards

M/15 Disodium phosphate, See page 128.

0.1% stock phenol red: 0.1 gm phenolsulfonphthalein sodium salt in 100 ml distilled water. Autoclave for storage in the refrigerator.

Prepare six screw-cap test tubes (16 x 125) as follows:

Tube No.	M/15 Na_2HPO_4	0.1% Phenol Red	Reading
1	4 ml	10 drops 0.1% stock	5+
2	4 ml	1 ml from tube 1	4+
3	3 ml	2 ml from tube 2	3+
4	3 ml	2 ml from tube 3	2+
5	3 ml	2 ml from tube 4	1+
6	3 ml	2 ml from tube 5	±

Discard 2 ml from tube 6, autoclave, seal, and store in the refrigerator. Color standard usually retains its color for 6 months.

Nitrate Reduction Standards

STOCK SOLUTIONS

1. M/15 disodium phosphate
2. M/15 monopotassium phosphate
3. M/15 trisodium phosphate
4. 1% phenolphthalein (1 gram in 100 ml 95% ethyl alcohol)
5. 1% brom thymol blue (1 gram in 100 ml 95% ethyl alcohol)

5A. 0.01% brom thymol blue: prepared by mixing 1.0 ml of stock No. 5 above in 100 ml distilled water.

WORKING BUFFER SOLUTION

Mix:

Stock No. 1 ..... 35 ml
Stock No. 2 ..... 5 ml
Stock No. 3 ..... 100 ml

PREPARATION OF STANDARDS

1. Line up eight clean test tubes in a rack. Use tubes the same size as for the test.
2. Put 2 ml of working buffer solution into seven tubes.
3. To 10 ml of working buffer solution, add 0.1 ml of stock No. 4 and 0.2 ml of stock No. 5A.
4. Add 2 ml of the step 3 solution to the 8th tube. This is the 5+ color standard.
5. To the first tube in the series of seven tubes, add 2 ml of the step 3 solution. Mix well and carry over 2 ml to the next tube. Continue to make serial dilutions, discarding 2 ml from the 7th tube.
6. The color standard:
   tube in step 4 = 5+
   tube 1 = 4+
   tube 2 = 3+
   tube 4 = 2+
   tube 5 = 1+
   tube 7 = ±

   These colors should range from pink (±) to purplish red (5+).
7. Autoclave tubes, seal, and store in a refrigerator.

APPENDIX D: PREPARATION OF REAGENTS AND MEDIA

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APPENDIX D:

PREPARATION OF REAGENTS AND MEDIA

1. General Rules for Preparation

For media of the best quality, use chemicals of certified purity, clean glassware, freshly distilled water, and follow the directions precisely. To avoid contaminating reagents and media:

(1) Keep the environment as clean as possible.
(2) Use an isolated area and minimize traffic in the preparation room.
(3) Swab the work surface with a disinfectant before dispensing sterile reagents and media.
(4) Clean the floor with a wet mop. This will keep dust down.
(5) If necessary, place ultrafilters over air intakes to reduce air contaminants.
(6) Use high-intensity ultraviolet light to sterilize air when room is *not in use.* However, *do not* leave media exposed to *any* light rays.
(7) Use sterile glassware and equipment.
(8) Observe strict aseptic technique. Flame flasks and tubes when adding enrichment or drugs to media.
(9) Do not leave covers off Petri dishes after pouring the media into plates.
(10) Be sure the Oleic acid Albumin Dextrose Complex (OADC enrichment) and drugs are properly sterilized before adding them to the sterile basal medium.
(11) Check pore size of sterilizing filters to make certain that they are adequate.
(12) Check temperature and pressure of autoclave.
(13) When preparing eggs to add to egg-base media, be sure that the shells of the eggs are carefully cleaned before breaking.

2. 0.2% Bovine Albumin Fraction V

(1) Dissolve 0.85 g sodium chloride in 100 ml of distilled water.
(2) Add 2.0 g of bovine albumin fraction V (2% solution). Dissolve by swirling flask or by gently stirring on a magnetic stirrer. Adjust pH to 6.8 with 4% NaOH.

(3) For 0.2% solution, dilute 1:10 with sterile saline.

3. **5% Oxalic acid**

Dissolve 50 g of oxalic acid in 950 ml of distilled water. Autoclave and store at 4°C.

4. **M/15 Phosphate Buffer Solutions**

Buffer solutions are prepared by mixing two stock solutions in proportions to give the required pH.

a. **STOCK SOLUTIONS**

(1) M/15 disodium phosphate: In a volumetric flask dissolve 9.47 g of anhydrous Na_2HPO_4 in distilled water to make one liter (1000 ml).

(2) M/15 monopotassium phosphate: In a volumetric flask dissolve 9.07 g of KH_2PO_4 in distilled water to make one liter (1000 ml).

b. **pH 6.8 BUFFER SOLUTION**

Mix 50.0 ml of (1) with 50 ml of (2).
Check on pH meter.

c. **pH 7.0 BUFFER SOLUTION**

Mix 61.1 ml of (1) with 38.9 ml of (2).
Check on pH meter.

d. **pH 6.6 BUFFER SOLUTION**

Mix 37.5 ml of (1) with 62.5 ml of (2).
Check on pH meter.

5. **Physiological Saline (0.85%)**

Dissolve 8.5 g sodium chloride in one liter of distilled water. Autoclave and store at room temperature.

6. **2.9% Sodium Citrate (0.1M)**

Dissolve 29.4 g of anhydrous Na citrate in one liter of distilled water. Autoclave and store at room temperature. If a precipitate forms, discard and prepare fresh solution.

7. **4% Sodium Hydroxide (1N)**

Dissolve 40 g of NaOH in one liter of distilled water. Autoclave and store at room temperature. If a precipitate forms, discard and prepare fresh solution.

8. **4% Sulfuric acid**

Add slowly, with stirring, 40 ml of sulfuric acid to 960 ml of distilled water. Store at 4°C.

9. **Zephiran-trisodium phosphate**

Dissolve 1 kg of trisodium phosphate ($Na_3PO_4 \cdot 12\ H_2O$) in 4 liters of hot distilled water. To this solution add 7.5 ml of Zephiran concentrate (17% benzalkonium chloride, Winthrop Laboratories). Mix well. Store at room temperature.

10. **Preparation of American Trudeau Society (ATS) Medium**

a. **INGREDIENTS**

Potato flour	20.0 g
Distilled water	490.0 ml
Glycerol	10.0 ml
Egg yolk	500.0 ml
1% malachite green in 50% alcohol	20.0 ml

b. **PROCEDURE**

(1) Combine potato flour, water, and glycerol in a 2-liter flask.
(2) Autoclave at 121°C for 30 minutes.
(3) Cool to 50°C.
(4) MEANWHILE: Prepare the fresh egg yolk as follows: Carefully clean fresh hen eggs.* Using aseptic techniques, separate the egg yolk from the white. For the 500 ml egg yolk, use a proportion of one whole egg to 11 yolks.
(5) Combine the homogenized egg yolk mixture with the cooled potato flour water.
(6) Add 20.0 ml malachite green. Mix thoroughly.
(7) Dispense 5-6 ml per tube into sterile screw-cap tubes.
(8) Slant and coagulate by inspissation at 85°C for 1 hour.

11. **Preparation of Modified Lowenstein Egg Medium**

(1) For salt solution combine:

Monopotassium phosphate (anhydrous)	2.4 g
Magnesium sulfate $\cdot 7H_2O$	0.24 g
Magnesium citrate	0.6 g
Asparagine	3.6 g
Glycerol (reagent grade)	12.0 ml
Distilled water	600.0 ml

*Fresh eggs, not more than 1 week old, are cleaned by scrubbing with a hand brush in a soap solution (about 5%). After scrubbing, the eggs are allowed to stand in the soap solution for 30 minutes. Rinse thoroughly in running water, then soak eggs in 70% alcohol for 15 minutes. With well scrubbed hands, break the eggs into a sterile flask and homogenize by hand shaking. Filter eggs through 4 layers of sterile gauze into a sterile graduated cylinder.

(2) Add potato flour ..... 30.0 g
(3) Autoclave at 121°C for 30 minutes.
Cool to room temperature.
(4) Add malachite green (2% aqueous solution freshly prepared) ..... 20.0 ml
(5) Add homogenized whole eggs ..... 1000.0 ml*
(6) Mix and pour into a sterile aspirator bottle or funnel with a bell attachment (test tube filling device) and dispense.
(7) Place approximately 6-8 ml medium into each 20 x 150 mm sterile screw-cap test tube.
(8) Slant tubes and coagulate by inspissation at 85°C for 50 minutes.
(9) Incubate at 37°C for 48 hours as a sterility check.
(10) Medium may be stored in the refrigerator for several months if caps are tightly closed to prevent evaporation.

12. **Preparation of 7H-10 Medium from Basic Ingredients**

Six stock solutions may be prepared in advance. When prepared and stored as indicated, these solutions remain stable for at least a month. If precipitation or flocculation occurs in any stock solution, discard and prepare fresh solutions.

a. **TO PREPARE THE SIX SOLUTIONS**

Solution No. 1

Monopotassium phosphate, A.C.S. (Anhydrous) ..... 15.0 g
Disodium phosphate, A.C.S. (anhydrous) ..... 15.0 g
Distilled water to make ..... 250.0 ml
Autoclave at 121°C for 15 minutes.
Store this solution at room temperature.

Solution No. 2

Ammonium sulfate, A.C.S. ..... 5.0 g
Monosodium glutamate (glutamic acid, monosodium salt) ..... 5.0 g
Sodium citrate $\cdot 2H_2O$, USP ..... 4.0 g
Ferric ammonium citrate ..... 0.4 g
Magnesium sulfate $\cdot 7H_2O$, A.C.S. ..... 0.5 g
Biotin (in 2 ml of 10% ammonium hydroxide) .... 5.0 mg
Distilled water to make ..... 250.0 ml
Autoclave at 121°C for 15 minutes.
Store in the refrigerator at 4°-10°C.

Solution No. 3

Calcium chloride $\cdot 2H_2O$, A.C.S. ..... 50 mg
Zinc sulfate $\cdot 7H_2O$, A.C.S. ..... 100 mg

Copper sulfate $\cdot 5H_2O$, A.C.S. ..... 100 mg
Pyridoxine hydrochloride ..... 100 mg
Distilled water to make ..... 100 ml
Store in the refrigerator at 4°-10°C.

Solution No. 4
Reagent grade glycerol. Store at room temperature.

Solution No. 5
Aqueous malachite green—0.01%
Dissolve 0.1 g malachite green in 100 ml distilled water.
Dilute 1:10 to make 0.01%.
Store at room temperature or 4°-10°C *in the dark.*

Solution No. 6
Oleate-Albumin-Dextrose-Catalase (OADC)
(1) Dissolve 50 g bovine albumin fraction V in 900 ml freshly prepared saline (0.85% NaCl).
(2) Add 30 ml sodium oleate prepared as follows:
N/20 NaOH ..... 30.0 ml
Oleic acid ..... 0.6 ml
Warm to 56°C and swirl gently to dissolve.
(3) Adjust pH to 7.0 with 4% NaOH.
(4) Heat in a water bath at 56 °C for 1 hour.
(5) Add 40 ml of sterile 50% solution of dextrose prepared as follows:
To 30 ml of boiling distill. water, add 25 g of dextrose (glucose). Stir to dissolve.
Add distilled water to make 50 ml total volume.
Autoclave 10 minutes at 121°C.
(6) Add 0.02 ml technical catalase.*
(7) Sterilize by membrane filter. To facilitate this process, use a pre-filter with the sterilizing membrane (0.45 micron) and filter while the albumin is still warm.
(8) Dispense in sterile screw-cap tubes in 20 ml or other convenient amounts.
(9) Incubate 24 hours at 35°-37°C. If sterile, the mixture is ready for use.
(10) Store in the refrigerator in air-tight containers. Do not freeze.

Note: *If it is inconvenient to prepare solution No. 6, the commercially available OADC is an acceptable substitute.*

*Catalase is inactivated by Seitz and sintered glass filters. If membrane filter sterilization is unavailable, omit the catalase from solution No. 6 and add it to the sterile completed medium.
To prepare sterile catalase, add 0.02 ml catalase to 20 ml 0.85% saline: this solution contains 1000 mcg/ml. Sterilize by membrane filter. To each 100 ml of completed medium, add 0.2 ml of the sterile catalase solution.

b. **TO PREPARE 800 ml COMPLETED MEDIA FROM THE SIX STOCK SOLUTIONS (OR FOUR "BATCHES" OF 200 ml EACH):**

(1) Distilled water . . . . . .680.0 ml
Solution No. 1 . . . . . 20.0 ml
Solution No. 2 . . . . . 20.0 ml
Solution No. 3 . . . . . 0.8 ml
Solution No. 4 . . . . . 4.0 ml
(2) Adjust the pH to 6.6 by adding approximately 0.4 ml of 6N HCl. Check on pH meter.
(3) Add solution No. 5 . . . . . 2.0 ml
(4) Divide into four equal parts of 180 ml each.
(5) To each 180 ml, add agar . . . . . 3.0 g
(6) Autoclave at 121°C for 10 minutes.
(7) Cool to 56°C (for drug media, cool to 50°-52°C).
(8) Add to each 180 ml flask:
(a) Solution No. 6 . . . . . 20.0 ml
(b) If catalase is not in OADC, prepare a sterile solution containing 1000 mcg/ml and add . . 0.4 ml (see footnote, previous page)
(c) For drug media, add sterile drug.
(9) Mix gently but thoroughly and dispense into Petri dishes. (20 ml per dish or 5 to 6 ml per quadrant in four-section dish). *Media must be cooled and dispensed within 1 hour after autoclaving.*
(10) Allow to solidify at room temperature *in the dark.*

13. **Preparation of 7H-10 Agar from Commercial Base**

Check the bottle label for this reference, "Acta Tuberc. Scand. *38*:66, 1960." This reference on the label indicates that the bottle contains the correct ingredients.

Note: *Experience has shown that boiling the basal medium before autoclaving results in a medium of inferior quality. This result may be avoided by preparing the medium in 200 ml amounts and autoclaving for 10 minutes.*

To prepare 200 ml complete medium follow these directions precisely:

(1) Suspend 3.8 g* of Middlebrook 7H-10 basal medium in 180 ml of freshly distilled water and add 1.0 ml of reagent grade glycerol.
(2) Swirl base into suspension and sterilize in the autoclave for 10 minutes at 121°C.

*Calculated from directions on the label for formula in Acta. Tuberc. Scand.

(3) Remove medium from autoclave *as soon as* the pressure will allow and place in a water bath at 50°-56°C.
(4) As soon as cooled to 50°-56°C, add 20 ml of OADC enrichment.
(5) For drug media, cool to 50°-52°C and then add the required amount of drug.
(6) Dispense *within 1 hour after autoclaving.*
(7) Allow to solidify at room temperature *in the dark.*

Note: *When the autoclaved medium is allowed to stand for more than 1 hour in the water bath, a precipitate begins to form. Experience has shown that this practice should be avoided. Pour as soon as possible. Always check the medium when taking it out of the autoclave. It should be clear and transparent. If a precipitate is present, it may be due to (a) sterilization temperature within the autoclave being reached too rapidly to dissolve the base in 10 minutes, (b) poor quality basal medium, and/or (c) dirty glassware.*

Caution: *Never autoclave 7H-10 medium base and store it in the refrigerator for future use. The heat required to melt it results in overheating and a medium of poor quality.*

For best results do not prepare in lots of more than 400 ml.

14. Preparation of Tween-Albumin Liquid Medium

a. MIDDLEBROOK 7H-9 BROTH

To prepare one liter (1000 ml) of medium:

(1) Combine 900 ml of distilled water with 0.5 g Tween 80 *or* 2 ml of glycerol as desired. Do not use Tween 80 and glycerol together.
(2) Add and dissolve the following salts in the order listed:

Ammonium sulfate .....0.5 g
(20 ml of 2.5% solution)

Glutamic acid, monosodium salt .....0.5 g
(20 ml of 2.5% solution)

Sodium Citrate $\cdot 2H_2O$ .....0.1 g
(1 ml of 10% solution)

Pyridoxine hydrochloride .....0.001 g
(1 ml of 0.1% solution)

Biotin .....0.0005 g
(0.25 ml of 0.2% solution)

Disodium phosphate (anhydrous) .....2.5 g

Monopotassium phosphate (anhydrous) .....1.0 g

Ferric ammonium citrate (green) .....0.04 g
(0.4 ml of 10% solution)

Magnesium sulfate $\cdot 7H_2O$ .....0.05 g
(5 ml of 1.0% solution)

Calcium chloride $\cdot 2H_2O$ .....0.0005 g
(0.5 ml of 0.1% solution)

Zinc sulfate $\cdot 7H_2O$ .....0.001 g
(1 ml of 0.1% solution)

Copper sulfate $\cdot 5H_2O$ .....0.001 g
(1 ml of 0.1% solution)

Adjust the pH of the solution to 6.6 using 10% HCl.

(3) Autoclave at 121°C for 20 minutes.

(4) Allow to cool to less than 50°C and add the following sterile enrichment solutions (for preparation see (6) below):

(a)	50% glucose	4 ml
(b)	Catalase (1000 mcg/ml)	2 ml
(c)	5% bovine albumin	100 ml

(5) Dispense in freshly sterilized screw-cap test tubes or other containers as desired. (Use 5 ml amounts in 20 mm diameter tubes.) Store in the refrigerator. To prevent evaporation during storage, tube caps must be securely tightened.

(6) Preparation of Solutions (a), (b) and (c) for step (4). (The commercial ADC contains these solutions and is a suitable substitute.)

(a) Prepare 50% glucose by dissolving 50 g glucose in 60 ml distilled water. To this add 1.0 ml of 10% citric acid. Autoclave for 10 minutes at 15 pounds pressure (121°C).

(b) Add 0.02 ml catalase (technical grade) to 20 ml of 0.85% NaCl in distilled water. Sterilize by membrane filtration. Prepare fresh each time needed.

(c) Mix 5.0 g of bovine albumin Fraction V and 95 ml of 0.85% NaCl in distilled water. Adjust pH

to 6.8 with either 50% NaOH or 16%-19% HCl, whichever is needed. Filter–sterilize the solution. Dispense in freshly sterilized screw-cap containers. Incubate at 37°C overnight to check for sterility. Place in a 56°C water bath for 30 minutes to inactivate lipase. Store at 4°C.

b. COMMERCIAL PRODUCTS

The Middlebrook 7H-9 and Dubos-Middlebrook Tween-albumin liquid media are satisfactory as supplied by commercial sources.

Prepare according to directions on the label. Tube in 5 ml quantities in 20 x 150 mm sterile screw-cap test tubes. Incubate for sterility check.
Liquid media may be stored in the refrigerator indefinitely, provided it is tightly capped and evaporation does not occur.

15. Sodium Chloride Tolerance

The control and sodium chloride medium should be prepared from the same "batch" of basal medium. Egg-base medium was used for the test results given in this manual.

a. AMERICAN TRUDEAU SOCIETY (ATS)

Divide the 500 ml distilled water-glycerol-potato flour into two equal portions of 250 ml each. To one portion add 25.0 g of sodium chloride. Autoclave, cool, and add 250 ml of egg yolk and 10 ml malachite green to each portion. Tube and inspissate as directed for ATS (p. 129).

b. LOWENSTEIN-JENSEN (L-J)

Divide the 600 ml salt-solution-glycerol (page 129) into two equal portions of 300 ml. To each portion add 15.0 g of potato flour. To one portion add 40.0 g of sodium chloride (this is 5% NaCl final concentration in the completed medium). The second portion is the control medium.

Autoclave, cool, and add 500 ml homogenized egg and 10 ml malachite green to each portion. Tube and inspissate as directed for L-J.

16. Storage of Media

(1) Always shield any media from direct daylight.
(2) Place plates in clean plastic bags or sterile dry cannisters.

(3) Before storage, tighten tops of screw-cap tubes.
(4) Media to be used within 1 week after preparation may be stored at room temperature *in the dark*.
(5) To prevent dehydration, any media more than 1 week old should be stored in the refrigerator.
(6) Properly stored media may be kept 2 to 3 months.
(7) Discard any unused media that shows signs of dehydration or contamination.

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